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Therapeutic Effect of Artificial Femoral Head Replacement and Proximal Femoral Nail Antirotation on Elderly Unstable Intertrochanteric Fractures

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Abstract

Objective: To compare the clinical efficacy of artificial femoral head replacement and Proximal femoral nail antirotation (PFNA) in the treatment of unstable femoral intertrochanteric fractures in the elderly. **Methods:** This study retrospectively analyzed 60 elderly patients with unstable intertrochanteric fractures treated with PFNA and artificial femoral head replacement from 2015.06 to 2018.06, of which 34 were in the PFNA group (Group A) and 26 in the artificial femoral head replacement group (Group B). Statistical analysis of relevant surgical indicators such as surgical time, intraoperative blood loss, postoperative blood transfusion, postoperative time to landing, postoperative infection rate, hospital stay, number of secondary operations, postoperative VAS score, and postoperative Hip function score comparison. **Results:** All 60 patients were followed up for 1 - 24 months. Compared with the artificial femoral head replacement group, the operation time of PFNA group was shorter, the blood loss during operation was less, and the difference was statistically significant ($P < 0.05$). The postoperative blood transfusion volume, postoperative time, hospital stay, and postoperative time were higher in PFNA group. In the bone replacement group, the postoperative VAS score and the Harris score were lower than those in the artificial femoral head replacement group, and the differences were statistically significant ($P < 0.05$). There was no significant difference in the incidence of postoperative infection and the number of secondary operations between the two groups ($P > 0.05$). **Conclusion:** The hip joint function and pain scores of the artificial femoral head replacement group in the early and follow-up periods are better than those of the PFNA group. The artificial femoral head replacement is

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more suitable for the treatment of elderly unstable intertrochanteric fractures.

Keywords

Artificial Femoral Head Replacement, PFNA, Elderly, Unstable Femoral Intertrochanteric Fracture, Efficacy Analysis

1. Background

Hip fractures in the elderly are one of the three major types of fractures in the elderly. According to related reports, 90% of patients with intertrochanteric fractures are older than 70 years, accounting for 45% of hip fractures, of which unstable types account for about 35% - 40% [1] [2], the mortality of femoral intertrochanteric fractures in the elderly is high, and the mortality rate of conservative treatment is 34.6% [3]. As China, Japan and other countries enter the aging society, 50% of hip fractures worldwide will occur in Asia in the future, and the incidence rate will be 2 - 3 times that of 2050 by now [4]. At present, the majority of scholars believe that the surgical treatment is better than the conservative treatment of intertrochanteric fractures, and that intramedullary fixation is the most effective treatment. However, for elderly patients with unstable intertrochanteric fractures, due to factors like serious fracture comminution which causes internal fixation instability, osteoporosis which makes it easy to be cut, even after intramedullary fixation, patients could not move in early stages. Besides, there are many complications. Nearly 33.3% of elderly patients with intertrochanteric fracture died within 6 months after fracture. As a result, nearly 33.3% of elderly patients with intertrochanteric fracture died within 6 months after fracture. Studies have shown that the use of artificial joint replacement in the treatment of elderly intertrochanteric fracture has a good effect; patients can be early out of bed activities, thus reducing the occurrence of bed-ridden complications. However, most scholars believe that the artificial femoral head replacement should not be used as one-stage operation for intertrochanteric fracture, but as a remedy or revision operation for failed internal fixation of intertrochanteric fracture. Therefore, what is more controversial for elderly patients with unstable intertrochanteric fractures is the choice of surgical method. In this study, a total of 60 elderly patients with unstable intertrochanteric fractures were treated in our hospital from 2015.06 to 2018.06. They were treated with PFNA and artificial femoral head replacement, respectively, and the effects of the two surgical methods were compared, as reported below.

2. Materials and Methods

2.1. General Information

This study randomly selected 60 elderly patients with unstable femoral intertrochanteric fractures treated with PFNA and artificial femoral head replacement

from 2015.06 to 2018.06. The inclusion criteria were: 1) age ≥ 70 years; 2) fractures according to AO classification unstable femoral intertrochanteric fracture; 3) Purely closed or without other serious injuries; 4) surgery completed within 48 or 72 hours after the injury; 5) the hip joint of the patient can walk with weight-bearing without significant pain before the fracture; 6) all the operation was performed by the same group of doctors .among them:

PFNA treated 34 cases (Group A), 8 males and 26 females, aged 70 - 82 (76 ± 5.9) years; fractures were classified by AO: 16 cases of type A2.2, 10 cases of type A2.3, and A3. There were 6 cases of type 1 and 2 cases of type A3.3; the osteoporosis index was −3.25 ± 0.51.

Artificial femoral head replacement was performed in 26 cases (group B), 7 males and 19 females, aged 71 - 82 (76.8 ± 5.9) years; fractures were classified by AO type: 17 cases of type A2.2, 4 of type A2.3 There were 3 cases of type A3.1 and 2 cases of type A3.3. The osteoporosis index was −3.25 ± 0.51.

The basic diseases include medical diseases such as coronary heart disease, hypertension, diabetes, and chronic heart failure. In order to compare the differences between the medical diseases of group A and group B, those with only one medical disease are classified as one category, and those with two medical diseases are classified as one category, the combination of 3 and more medical diseases as a category. There were no significant differences in gender, age, fracture classification, medical complications, and osteoporosis between the two groups ($P > 0.05$).

2.2. Preoperative Preparation

Before surgery, the patients in both groups had perfect head, heart, lower limb blood vessels and other examinations to clarify the basic conditions of the patients; please refer to relevant specialists for diagnosis and treatment of related medical diseases; if oral anticoagulants are used, the drug should be discontinued according to the instructions; antibiotics should be given intravenously 30 minutes before surgery; to prevent excessive bleeding during surgery, routine blood preparation before surgery.

2.3. Surgical Methods

PFNA treatment group (Group A): the patient was in a supine position, the affected foot was placed on a traction foot frame, the limbs flexed and abducted on the healthy side, and the perineum was placed against the traction column to protect the perineum. The pelvis is placed in a horizontal position. The traction bed is closed after the traction is closed and the affected limb is satisfied. The traction bed is fixed. A 6 cm incision is made proximally at a height of 1 - 2 cm above the apex of the greater trochanter. Muscle tissue is bluntly isolated in the direction of the muscle fibers of the middle muscle. After exposing the apex of the greater trochanter, insert the guide needle into the medullary cavity with the help of an opener and "golden fingers". C-arm fluoroscopy confirms the posi-

tion of the guide needle. See depth for perspective. Under the guidance of the sight, adjust the forward inclination angle, screw in the guide pin, and confirm that the guide pin is inserted into the femoral neck and femoral head. After the position and depth are satisfied, insert a suitable spiral blade and pressurize the broken end. Partial free bone mass was left open. The distal femoral shaft lock is inserted through the distal aiming system and sutured layer by layer after irrigating.

Artificial femoral head replacement treatment group (Group B): the patient adopted the surgical approach of the lateral position and exposed the femoral neck and the trochanter according to the approach. After the conventional osteotomy of the femoral neck, the osteotomy surface and the intertrochanteric fracture line were removed. The femoral head was taken out after the bone was removed, and a part of the small trochanter was left open. After the medulla reamed, the appropriate type of long-stem biological prosthesis was placed. After the long-stem prosthesis was successfully inserted, some patients used steel cables to fix the fracture block between the rotors through the reserved holes in the prosthesis stem. After installing an appropriately sized artificial femoral head, the hip joint function meets the basic requirements, and the incision can be sterilized, a negative pressure drainage device is placed, and an analgesic "cocktail" is injected around the incision, and the incision is closed layer by layer.

2.4. Postoperative Management

Antibiotics were routinely applied to prevent infection in the two groups for 48 to 72 hours after operation. Low molecular weight heparin and plantar pumps were used to prevent lower extremity venous thrombosis, oral analgesics, and routine fluid replacement. The blood analysis, CRP and other indicators were reviewed on the first and third days after operation, and if there was any abnormal symptomatic treatment. On the first day, functional exercises on the bed began. Group A performed weight-bearing exercise 2 or 3 weeks after surgery. In Group B, standing weight-bearing exercises can be performed with the aid of a walker for 3 days after surgery.

2.5. Comparative Evaluation Index

Related surgical indexes including surgical time, intraoperative blood loss, postoperative blood transfusion, postoperative landing time, postoperative infection rate, hospital stay, number of secondary operations, postoperative VAS score, and postoperative Harris score were compared between the two groups.

2.6. Statistical Methods

SPSS 24.0 statistical software was used for analysis. Measurement data were expressed as mean ± standard deviation ($x \pm s$) and independent sample t test was used. Count data were expressed as percentage and χ^2 test was used. Differences

were statistically significant at $P < 0.05$.

3. Results

All 60 patients were followed up for 1 - 24 months, with an average of 15 months. Specific comparison indicators are shown in Tables 1-4 below.

Table 1. Comparison of preoperative clinical data between PFNA group (Group A) and artificial femoral head replacement group (Group B).

Group	Number of cases (example)	Male/female (example)	Age	AO typing		Osteoporosis index
				A2	A3	
Group A	34	14/20	76 ± 5.9	26	8	2.64 ± 0.12
Group B	26	11/15	76.8 ± 5.9	21	5	2.64 ± 0.12

Table 2. Surgical indicators of PFNA group (Group A) and artificial femoral head replacement group (Group B).

Group	Operation time (min)	Intraoperative blood loss (ml)	Postoperative blood transfusion (ml)
Group A	46.3 ± 11.4	90.0 ± 20.0	200 ± 200
Group B	62.2 ± 10.5	150.0 ± 35.0	200 ± 200
t value	−10.14	−13.37	2.33
p-value	<0.05	<0.05	<0.05

Table 3. Related indexes after operation in PFNA group (Group A) and artificial femoral head replacement group (Group B).

	Landing time after operation (days)	Postoperative infection rate (%)	Length of hospital stay (days)	Second operation (example)
Group A	21.0 ± 7.0	2.94	14.0 ± 6.0	1
Group B	7.0 ± 2.0	0	12.0 ± 5.0	0
T value	7.24	0.873	2.41	0.873
p-value	<0.05	>0.05	<0.05	>0.05

Table 4. Postoperative pain and function indexes of PFNA group (Group A) and artificial femoral head replacement group (Group A).

Group	Postoperative VAS score (points)				Harris score after operation (points)			
	1 month	3 months	6 months	12 months Rear	1 month	3 months	6 months	12 months Rear
Group A	8.2 ± 1.8	5.2 ± 1.7	3.2 ± 1.2	2.2 ± 1.1	61.0 ± 7.0	71 ± 7	84 ± 7	88 ± 6
Group B	7.1 ± 1.6	4.3 ± 1.8	2.1 ± 1.3	1.1 ± 0.5	81 ± 6	86 ± 7	90 ± 7	91 ± 6
t value	7.07	3.96	6.27	9.55	−22.23	−15.07	−4.95	−4.09
p-value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Compared with the artificial femoral head replacement group, the operation time of PFNA group was shorter, and the blood loss during operation was less, and the difference was statistically significant ($P < 0.05$). The postoperative blood transfusion volume, postoperative time, hospitalization time, and duration of PFNA group were higher than those of artificial femoral head. In the bone replacement group, the postoperative VAS score and the Harris score were lower than those in the artificial femoral head replacement group, and the differences were statistically significant ($P < 0.05$). There was no significant difference in the incidence of postoperative infection and the number of secondary operations between the two groups ($P > 0.05$).

4. Discussion

With people's emphasis on quality of life and new surgical methods and clinical application of internal fixation devices, most elderly patients and their families tend to choose surgical treatment. The purpose of surgical treatment is: strong fixation, early movement. The shortcomings of the external fixator are more prominent, it cannot bear the weight early, it is inconvenient to carry, the nail channel is loose and infected, and the bone fracture deformity heals and delays healing in the later stage [5]. Therefore, with the exception of patients with extremely poor systemic conditions that cannot tolerate anesthesia, external fixators should be used with caution. Extramedullary fixation belongs to eccentric fixation and has poor anti-rotation ability. For unstable intertrochanteric fractures, internal fixation can easily lead to fatigue fracture due to internal wall defects. Postoperative rehabilitation cannot be carried out with early weight bearing [6] [7]. Intramedullary fixation conforms to the characteristics of human biomechanics, has the advantages of standardized operations, less trauma during surgery, and better stability [8], and has unparalleled advantages of extramedullary fixation.

PFNA is an intramedullary fixation device designed for femoral intertrochanteric fractures. Due to the closed reduction of the small incision, the intraoperative trauma is small [9], and the short and central fixation of the arm [10] does not require anatomical reduction, which fully protects the bone. Periplasmic blood transport has unparalleled advantages of ordinary internal fixation, but due to severe osteoporosis in elderly patients, the initial stability of PFNA is not enough to carry weight early [11].

Early femoral head replacement was an internal fixation device specifically designed for elderly femoral neck fractures [12]. In 1974, Tronzo's first attempt to use artificial joint replacement to treat femoral intertrochanteric fractures received widespread attention [13]. According to Sing *et al.* [14] doctors realized the advantages of artificial femoral head replacement and used it in the treatment of elderly femoral intertrochanteric fractures, which achieved good clinical results. Zhang Hua *et al.* [15] thought that after the artificial femoral head replacement surgery, a better hip joint stability was obtained immediately, the hip

joint could be partly moved immediately, and the rehabilitation exercise was carried out under the weight to reduce postoperative complications.

With the development of artificial femoral head replacement and ERAS in the lateral lying DAA, the surgical injury is getting smaller and smaller, and the weight bearing time of patients is getting earlier and earlier [16]. The lateral lying DAA is directly separated from the muscle space to reveal the fractured end. There is no need to cut muscles during the operation. The soft tissue damage is small, which reduces the postoperative pain of the patient [17] [18].

PFNA surgery requires reduction with the aid of a traction table and a C-arm machine. Due to the complexity of the fracture, it is often accompanied by the separation of the large and small trochanters and the separation of the femoral neck shaft. Reduction is relatively difficult. The artificial femoral head replacement is a fixed operation, which does not require reduction during the operation, without the need for a traction bed and a C-arm machine. The surgeon has a short learning curve, is easy to grasp, and has no radiation exposure, which is beneficial to the relatively basic hospital.

Long-stem PFNA can be replaced in patients with secondary fractures after PFNA. At the same time, artificial femoral head replacement is also an effective method to save secondary fractures [19]. With the emergence of long-stem and full-stem joint prostheses, the treatment of fractures around the prosthesis after artificial femoral head replacement has become easier and more convenient [20], and the second fracture rate is only 0.1% - 2.1% [21].

Immediately after the artificial femoral head replacement, the hip joint has better stability. The hip joint can be partly moved immediately. With the application of local analgesics, the hip pain is significantly reduced, so the hip joint function and pain scores early after surgery. All were better than PFNA group. The artificial femoral head replacement group can use a walker to stand weight-bearing exercises early, reduce complications caused by long-term bed rest, and help improve the overall situation. During follow-up, patients' hip joint function and pain scores were better than PFNA group. Due to the limitation of follow-up time, the long-term effect needs to be further verified.

Unstable intertrochanteric fractures in the elderly are often accompanied by severe osteoporosis and a series of medical diseases. The treatment is difficult, and conservative treatment requires long-term bed rest, which greatly increases the incidence of lower limb venous thrombosis and pulmonary infection, the incidence of Bedsore and other complications. The principle of surgical treatment is strong internal fixation and early functional exercise. Because of the complexity of unstable intertrochanteric fractures in the elderly, it is very important to choose the operative method. The type of fracture, the age of the patient, the basic condition of the patient and the economic condition of the Family should be considered before the operation. The aim of this study was to analyze and compare the clinical efficacy of artificial femoral head replacement and PFNA in the treatment of unstable intertrochanteric fractures in the elderly. All cases were

followed up and analyzed statistically. There were significant differences in operative time, intraoperative blood loss, postoperative blood transfusion, postoperative time, postoperative infection rate, hospital stay, number of secondary operations, VAS score and Harris Score. In general, artificial femoral head replacement is relatively simple and stable in the treatment of unstable intertrochanteric fractures in the elderly compared with PFNA, and can be performed early after operation, it is more suitable for the treatment of unstable intertrochanteric fractures in the elderly. Because of the small sample size, the short observation time and the fluctuation of the late patient data collection, the comparison of the two methods needs to be further studied in the future.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Effects of Occupational Noise on the Hearing Ability of Outpatient Clinic Nurses in Different Classes of Hospitals in Guangzhou

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Abstract

Background: Occupational noise can induce hearing impairment. Work-related hearing impairment has become a growing threat to medical practitioners who feel anxious about occupational noise exposure and its health outcomes or even experience auditory dysesthesia (including drumming, distending pain in the ears, and otalgia) after long-term exposure to a noisy work environment. **Objective:** To investigate the effects of occupational noise on the hearing ability of outpatient clinic nurses in Grade III, Level A and Grade II, Level A hospitals in Guangzhou. **Methods:** During June 2019, noise monitoring was performed by quantifying the noise levels at four measuring points (reception, waiting area, hallway, blood-sampling room) in five Grade III, Level A and five Grade II, Level A hospitals, in Guangzhou, four times a day (8 AM, 10 AM, 2 PM, and 4 PM) for 19 working days, using a professional noise measuring application for smartphones. The measurements were verified and used to create a database in Excel. Data analysis was conducted using SPSS22.0, and questionnaires were distributed to nurses who had been working at outpatient clinics for five years and above to assess the impacts of occupational noise exposure on the hearing ability. **Results:** In the Grade III, Level A hospitals, the sound levels at the four measuring points during the specific time periods were 4.92 - 6.75 dB above the permissible limit of 55 dB and were all significantly higher than the sound levels at the outpatient clinics of the Grade II, Level A hospitals ($P < 0.001$). Outpatient clinic nurses who had been serving with the Grade III, Level A hospitals for at least five years ($n = 140$, mean age: 44 ± 5.1) had a prevalence ratio of auditory dysesthesia higher than their counterparts ($n = 110$, mean age: 42 ± 6.1) in the Grade II, Level A hospitals ($P < 0.01$). **Conclusions:** Exposure to excess noise can lead

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to auditory dysesthesia in outpatient clinic nurses. Compared to those from Grade II, Level A hospitals, outpatient clinic nurses from Grade III, Level A hospitals are at higher risk of auditory dysesthesia. The noise levels at outpatient clinics should be closely monitored, and effective measures should be taken to reduce occupational noise exposure. Outpatient clinic nurses should enhance protective measures and receive preventive health exams on a regular basis.

Keywords

Noise, Outpatient Clinic Nurse, Auditory Dysesthesia, Survey

1. Introduction

Hazardous occupational noise is a public health threat. Occupational noise brings gradual damage to the hearing system and is particularly linked to hearing impairment. Existing studies on noise-induced hearing impairment largely focus on workers in the manufacturing industry, with only very few reports covering the impacts of occupational noise exposure on medical practitioners. As the medical and health sector develops, occupational safety and health are considered an increasingly important research interest.

In the recent five years, anxiety grows in hospitals as nurses have experienced auditory dysesthesia (including drumming, distending pain in the ears, and otalgia) closely associated with day-to-day exposure to occupational noise even though relevant symptoms are usually relieved after they leave their workplaces. Studies have suggested that there is a clear link between occupational noise-induced hearing loss and noise intensity, age, and length of service [1] [2]. In China, researchers mainly focus on occupational noise exposure in enterprises. For instance, Chen *et al*. [3] [4] [5] pointed out that workers in such economic subsectors as automobile manufacturing, site operation, and textile industries were high-risk populations for noise-induced hearing impairment and cardiovascular problems; of the workers having been exposed to occupational noise for five years, 36.04% were diagnosed with hearing impairment; those who had been employed for over five years were more likely to have hearing problems, blood pressure conditions, and abnormal ECG patterns. A foreign study suggested [6] that both patients and medical practitioners could be severely affected by noise pollution in hospitals. Chinese researchers largely emphasize the physiological and psychological impacts on patients exposed to a noisy hospital environment. Wang *et al*. [7] [8] [9] [10] showed that a noisy environment had a strong impact on a patient's emotions and could raise the risk of anxiety by up to 91.07%. Despite all that, noise-induced auditory dysesthesia in medical practitioners is rarely reported. To fill the gap, we carried out a one-month investigation concerning occupational noise and distributed questionnaires to nurses who had been working at the same outpatient clinics for at least five years for

self-evaluation of auditory dysesthesia (including drumming, distending pain in the ears, and otalgia). The results are reported as follows:

2. Subjects and Methods

2.1. Subjects

Five Grade III, Level A and five Grade II, Level A hospitals were selected randomly for noise monitoring at their outpatient clinics. Questionnaires were distributed to 250 nurses who had been working at these outpatient clinics for at least five years, including 140 from the Grade III, Level A hospitals (range: 35 - 56; mean age: 44 ± 5.1) and 110 from the Grade II, Level A hospitals (range: 28 - 54; mean age: 42 ± 6.4).

2.1.1. Hospitals

Five Grade III, Level A hospitals with at least 2000 outpatient visits per day and five Grade II, Level A hospitals with 400 to 700 outpatient visits per day were chosen for noise monitoring when the ambient temperature was 22˚C - 26˚C and the relative humidity was 50% - 70%.

2.1.2. Inclusion Criteria

Nurses were eligible for inclusion if they 1) had been working at the same outpatient clinics for at least five years, and 2) denied having a familial history of hearing loss, known ototoxic drug usage, excessive use of earphone, preexisting otological problems, known exposure to organic solvents, viral infection/vascular disease-induced inner hair cell dysfunction or physiological and structural damage to the inner ear, and any other underlying conditions. This study has obtained informed consent from all participants.

2.2. Methods

2.2.1. Noise Monitoring

Noise monitoring was performed using a professional sound level meter application (iDOBOOKER, Beijing, China) at four measuring points (reception, waiting area, hallway, blood-sampling room) in each of the 10 hospitals, four times a day (8 AM, 10 AM, 2 PM, and 4 PM) for 19 working days. Smartphones were installed with the noise measuring application and placed about 1.5 m away from windows, 1.2 - 1.5 m above the ground, and at least 1 m away from other reflecting surfaces. Parallel measurement (three times in total) was made at every measuring point during each period (1 min). For each measuring point, there were 12 measured values as the sound level during each period was measured three times. On this basis, a total of 3040 measured values were obtained after 19 working days, and the sound level at every measuring point was averaged over the specific period.

2.2.2. Questionnaire Survey

Questionnaires were randomly distributed to 140 outpatient clinic nurses from

the Grade III, Level A hospitals and 110 from the Grade II, Level A hospitals. The sample size of this survey was determined using formula $n = Z^2\sigma^2/d^2$. We used the confidence interval of 95%, the significant level of 0.05, the power of 0.8, and the overall standard deviation of 0.5, which were substituted into the formula to reach the minimum sample size as 97. The questionnaire is designed independently and has passed the assessment of reliability and validity of expert judgment. The questionnaire asks details about age, sex, length of service, whether the respondent has a familial history of hearing loss, known ototoxic drug usage, excessive use of earphone, preexisting otological problems, known exposure to organic solvents, viral infection/vascular disease-induced inner hair cell dysfunction or physiological and structural damage to the inner ear, and any other underlying conditions, whether the respondent experiences auditory dysesthesia (including drumming, distending pain in the ears, and otalgia) and if so, whether such symptoms are relieved after the respondent leaves the workplace. (This questionnaire survey focuses on the self-evaluation of auditory dysesthesia. Hearing exam will be performed in the follow-up study).

2.3. Indicators

2.3.1. Noise

GB 3096-2008 Environmental Quality Standard for Noise and GB 22337-2008 Emission Standard for Community Noise. China Environmental Science Press (Beijing) [11].

2.3.2. Nurses' Subjective Experience

Whether they experienced auditory dysesthesia (including drumming, distending pain in the ears, and otalgia) and if so, whether such symptoms were relieved after they left their workplaces.

3. Statistical Analysis

All statistics were registered, and a database was created and verified via Excel. SPSS22.0 was used for data analysis. Measurement data were expressed in the form of "mean ± standard deviation (x ± s)"; enumeration data were presented by percentage (n%). The *t*-test and the chi-squared test were applied in the data analysis, with $P < 0.05$ being considered statistically significant.

4. Results

As shown in Table 1, there is no statistically significant difference between the outpatient clinic nurses from Grade III, Level A and Grade II, Level A hospitals in age, length of service, and length of outpatient service, which indicates a high degree of comparability.

Table 2 suggests that at all measuring points, the outpatient clinics of the Grade III, Level A hospitals have higher sound levels compared to the outpatient clinics of the Grade II, Level A hospitals, and the differences are statistically significant. In other words, the outpatient clinics of the Grade III, Level A hospitals

Table 1. Demographic characteristics of nurses from the Grade III, Level A and Grade II, Level A hospitals.

Demographic Characteristics	Grade III, Level A (n = 140/100%)	Grade II, Level A (n = 110/100%)	*t*-value	*p*-value
Age	43.76 ± 5.11	42.38 ± 6.16	1.936	0.054
Length of service	23.63 ± 6.07	22.73 ± 7.15	1.076	0.283
Length of outpatient service	9.67 ± 4.36	10.10 ± 5.38	0.698	0.486

Table 2. Sound levels (dB) at the measuring points in the Grade III, Level A and Grade II, Level A hospitals.

Area	Grade III, Level A Hospital	Grade II, Level A Hospital	*t*-value	*p*-value
Reception	60.78 ± 4.87	53.36 ± 3.46	13.520	<0.001
Waiting area	61.75 ± 4.98	53.76 ± 3.30	14.507	<0.001
Hallway	59.92 ± 6.64	52.98 ± 3.49	9.934	<0.001
Blood-sampling room	59.37 ± 5.19	52.48 ± 2.39	12.482	<0.001

are faced with a greater noise challenge than those of the Grade II, Level A hospitals.

Table 3 reveals that compared to the Grade II, Level A hospitals, the Grade III, Level A hospitals have a higher proportion of outpatient clinic nurses who experience auditory dysesthesia (including drumming, distending pain in the ears, and otalgia), and the difference shows statistical significance. Further, about 66.43% of the outpatient clinic nurses from the Grade III, Level A hospitals report relief of relevant symptoms after work, which indicates that occupational noise is closely associated with the occurrence of auditory dysesthesia in these nurses.

5. Discussion

5.1. Definition of Noise and Relevant Standards

Noise [12] is defined as a sound produced by industrial production, construction, transportation or social life that causes disturbance to the surrounding living environment. Environmental noise pollution means the phenomenon where the noise level exceeds a specific threshold and causes disturbance to people's everyday life, work and study.

Threshold Noise Level in Urban Environment (dB).

Category	Daytime	Night-time
0	50	40
1	55	45
2	60	50
3	65	55
4	70	55

Table 3. Manifestations of auditory dysesthesia in outpatient clinic nurses from the Grade III, Level A and Grade II, Level A hospitals.

Manifestations	Grade III, Level A (n = 140/100%)	Grade II, Level A (n = 110/100%)	χ^2	*p*-value
Drumming	36/25.71%	0/0.00%	33.04	<0.01
Distending pain in the ears	36/25.71%	0/0.00%	33.04	<0.01
Otalgia	30/21.43%	0/0.00%	26.79	<0.01
Relief of symptoms (if any) after work	93/66.43%	-	-	-

Outpatient clinics and other medical spaces are subject to Category 1, that is, the sound level should remain below 55 dB during the daytime and 45 dB in the evening. According to the applicable national standards, noise in daytime means the sound level in such areas beyond the permissible limit of 55 dB [13].

5.2. The Influence of Different Levels of Occupational Noise on Clinic Nurses

The two groups of outpatient clinic nurses from the Grade III, Level A and Grade II, Level A hospitals are highly comparable in the light of the fact that they have no significant difference in age, length of service, and length of outpatient service. After noise monitoring for 19 working days, it was found that the average daytime sound levels at the four measuring points of the five Grade III, Level A hospitals were as follows: waiting area 61.75 ± 4.98 dB > reception 60.78 ± 4.87 dB > hallway 59.92 ± 6.64 dB > blood-sampling room 59.37 ± 5.19 dB; the average daytime sound levels at the four measuring points of the five Grade II, Level A hospitals were as follows: waiting area 53.76 ± 3.30 dB > reception 53.36 ± 3.46 dB > hallway 52.98 ± 3.49 dB > blood-sampling room 52.48 ± 2.39 dB. Clearly, the average sound levels at the four measuring points of the five Grade III, Level A hospitals during the 19 working days were all above the daytime limit of 55 dB, whereas none of the five Grade II, Level A hospitals exceeded the given threshold. The outpatient clinics of the Grade III, Level A hospitals are noisier than those of the Grade II, Level A hospitals probably because they have a greater number of daily outpatient visits, inadequate outpatient settings, and larger patient flows. Studies have shown that the proportion of outpatient clinic nurses from Grade III, Level A hospitals who suffer from auditory dysesthesia is 72.86% higher than that of Grade II, Level A hospitals, which provides support for concluding that long-term occupational noise exposure predisposes nurses to auditory dysesthesia.

5.3. Reports on Noise Health Effect(s)

A survey regarding noise and urban dwellers' health suggests that the incidence of hypertension increases by 3% when the noise level in an area is up by 1 dB. This is in agreement with the findings of Pang *et al.* [14] [15] [16] [17], which

revealed that long-term exposure to high-level noise could lead to hearing impairment and nonspecific injury of the cardiovascular system. Ryherd *et al*. [18] [19] pointed out that working in a noisy environment for a long time might cause dizziness, memory loss, irritability, fatigue, and low work efficiency; also, it could affect interpersonal communication and lower the energy level. When this occurs in medical practitioners, they may make mistakes in diagnosis and treatment. Sometimes loud noises can cause discomfort. Studies [20] [21] showed that most nurses did not fully understand noise health outcomes and their knowledge of noise largely varied because of sex, age, and length of service; if not keenly aware of the importance of self-protection against occupational noise at the beginning, they might face serious consequences. In this study, the incidence rates of drumming, distending pain in the ears, and otalgia in the outpatient clinic nurses from the Grade III, Level A hospitals were 25.71%, 25.71%, and 21.43%, respectively, while 66.43% reported relief of relevant symptoms after leaving the workplaces, which is significantly higher than the proportion of symptom relief in the Grade II, Level A hospitals and implies that for outpatient clinic nurses in Grade III, Level A hospitals, occupational noise is probably an important risk factor of auditory dysesthesia. A heightened awareness of self-protection and early prevention and control play a critical role in preventing occupational noise-induced hearing loss [22]. Therefore, hospitals should implement effective noise control to protect patients and medical practitioners.

6. Conclusions

Exposure to excess noise can lead to auditory dysesthesia in outpatient clinic nurses. Compared to those from Grade II, Level A hospitals, outpatient clinic nurses from Grade III, Level A hospitals are at higher risk of auditory dysesthesia. The noise levels at outpatient clinics should be closely monitored, and effective measures should be taken to reduce occupational noise exposure. Outpatient clinic nurses should enhance protective measures and receive preventive health exams on a regular basis.

This study has its limitations as other risk factors of auditory dysesthesia that may affect the study results have not been fully discussed.

Further study is needed for comparative analysis of auditory dysesthesia in nurses when multiple measures are taken to reduce noise exposure in the outpatient clinics of Grade III, Level A hospitals.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental

Table S1. Results of expert judgment (n = 12).

Questionnaire	Completely feasible (#/%)	Strongly feasible (#/%)	Feasible (#/%)	Weakly feasible (#/%)	Infeasible (#/%)
Content validity	4/33.33	5/41.67	3/25.00		
Construct validity	4/33.33	6/50.00	2/16.67		

Table S2. Results of test-retest reliability (two weeks after the preceding questionnaire survey, n = 50).

	N	R	P
Questionnaire	50	0.88	P < 0.01

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Autophagy-Inducing Effect of Compound Berberine on CNE2 NPC Cells via Interference with the Targets in P13K/AKT/mTOR Signaling Pathway

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Abstract

Objective: To investigate the autophagy-inducing effect of Compound Berberine (CBBR) on CNE2 nasopharyngeal carcinoma (NPC) cells and its possible targets in P13K/AKT/mTOR signaling pathway. **Methods:** CNE2 cells at exponential growth phase were taken as the target cells in this study. Firstly, IC50 concentration for CBBR was determined by MTT assay. Then, 3 different concentrations of CBBR, 0.25 mg·mL^{-1}, 0.50 mg·mL^{-1} and 1.00 mg·mL^{-1}, around the concentration of IC50, were taken for followed intervention experiments respectively. Fluorescein labeling method was utilized to assay the inducing effect of CBBR on the autophagic activity of CNE2 cells, followed by Western blot procedure to explore the changes of key messenger molecules in the autophagy-related signaling pathway of P13K/AKT/mTOR, both combined with 3-MA block test in a comparative way and carried out by detecting the expressive levels of Beclin 1, LC3-II and LC3-I as well as the ratio of LC3-II:LC3-I. **Results:** IC50 of CBBR was determined at the level of 0.5 mg·mL^{-1}. The inducing effect of CBBR on autophagy of CNE2 cells was shown occurring in various modes, not a simple concentration-dependent tendency, with its effect minimal at the concentration of 0.25 mg·mL^{-1} and maximal at the concentration of 0.50 mg·mL^{-1}, while only slightly higher at the concentration of 1.00 mg·mL^{-1} than that of 0.5 mg·mL^{-1}. Although its inducing effect was weakened a little following the pretreatment by 3-MA, the

effect combined with CBBR was still significantly higher than that of simply blocked by 3-MA. Moreover, changes in the expressive levels of Beclin1, LC3-II and LC3-I as well as LC3-II:LC3-I all showed a tendency corresponding to the changed autophagic features of CNE2 cells ($P < 0.05$ or $P < 0.01$), given more supporting evidences for the effect of CBBR on autophagy of CNE2 cells. **Conclusions:** CBBR can bring about inhibiting effect on the proliferating activity of CNE2 cells through inducing increased autophagic activity via intervening targets in P13K/AKT/mTOR signaling pathway, and this effect could not be completely blocked by the antagonist 3-MA.

Keywords

NPC Cells, Compound Berberine, Autophagy, Signaling Pathway, Intervening Effect

1. Introduction

There are multiple forms of tumor cell death such as necroptosis [1] [2] [3], pyroptosis [4] or inflammatory necrosis [5], autophagy [6] [7] [8], oncosis [9], entosis [10] [11], paraptosis [12], ferroptosis [13] [14] [15] and so on, with necrosis and apoptosis being the most commonly seen ones [16]. Each of these forms occurs with a different mechanism and holds various impacts on their biological effects, with that of autophagy being a very special one.

Autophagy is a kind of inherent endogenous mechanism for self-degradation of sub-cellular fractions within all kinds of eukaryotic cells [17], being a kind of phenomena with highly conservative characteristics within living organisms including xenophagy [18] and being crucial for the quality control mechanisms and maintenance of cellular homeostasis in various stem cells as well [19]. Under some stress conditions such as hunger, eukaryotic cells can survive by necessary energy supply guaranteed through such a very important biological event of "self-digestion" to maintain a continuous process of intracellular material metabolism in a turnover way, during which proteins and other components of damaged, senescent and dead organelles are degraded by enzymatic degradation of lysosomes and then being transformed into energy supply to the cells to help themselves get through the crisis caused by such stress stimulating events. This kind of mechanism can also be utilized to block damage and apoptosis inducing effects on cells themselves by degrading some toxic substances within them. Therefore, this mechanism has been well known as cellular "sweeper" or "cleaner". Autophagy can widely occur in various kinds of pathophysiological processes among eukaryotic cells, including developing, aging, neurodegenerative disorders, carcinogenesis and some kinds of infectious diseases. So, autophagy is closely associated with the several development processes and many kinds of diseased progress. However, autophagy holds a special feature of "dual-

ity" at some stages of illness or under some stress conditions, just like a rapier or "double-edged sword". In this aspect, carcinogenesis can be taken as the most typical sample associated with such an effect, that was discovered earliest. Increasing evidences are showing that autophagy plays very important roles in the process of tumorigenesis and in the response of tumor cells to anti-tumor therapies so that research in this field is becoming one of the hot subjects.

It has been confirmed that abnormality in autophagy can lead to transformation of normal cells into malignant ones via inducing gene mutation. Meanwhile, abnormal changes in the mechanisms of autophagy may induce, even exacerbate, the resistance of tumor cells to anti-tumor therapies and lead them to escape from the attack of anti-tumor therapies. On the other hand, synthesized therapeutic efficiency of anti-tumor therapies may be improved largely once tumor cells are successfully transformed into the process of apoptosis, or necrosis, via the pathway of autophagy inducing effect to blocking its protective effect on the survival of tumor cells.

Based on such a consideration, this work was aimed at exploring the intervening effect of Compound berberine (CBBR) on autophagic activity of NPC cells and its associated mechanism possibly with the signaling pathway of autophagy, because it has been confirmed through a series of previous work that CBBR held very strong inhibitory effects on the proliferating activity of NPC cells via apoptosis inducing action by both of concentration-dependent and time-dependent modes, and that it can intervene their potentiality of invading and migrating [20] [21] [22] [23].

2. Materials and Methods

2.1. Materials, Reagents and Equipment

1) Cell line Human nasopharyngeal carcinoma cell line CNE2 was bought from Cell Center of Basic Medicine Institute, China Academy of Medical Science.

2) Drug and reagents CBBR was extracted in our own laboratory from compound rhizoma coptidis formula based on a standardized operation program as a kind of compound aqueous extract, mainly composed of such ingredients as berberine, astragalin and flavone glycoside of *Hedyotis diffusa*, being different with the ethyl acetate extract fraction as reported before [24]. Rabbit antibodies against human LC3, Beclin1, PI3K, p-AKT, p-mTOR, HRP labeled second rabbit antibodies, and kits for PI3K/AKT/mTOR signaling pathway detection were all bought from CST Company. Rabbit antibody against human GAPDH was bought from Abcam Company, and 3-MA were bought from Shanghai Yuanye Company.

3) Equipment Inverted microscope (CKX31) was the product of Olympus Company, CO_2 incubator (MCO-50) was the product of Sanyo Company, and Fully Automatic Enzyme Label Analyzer (ELX800) was the product of BioTek Company.

2.2. Methods

1) All cell cultures were carried out routinely in an incubator with 5% CO_2 at 37˚C and saturated humidity.

2) Inhibitory effect of CBBR on the proliferating activity of CNE2 cells CNE2 cells at exponential growth phase in the culturing system of 1640 medium, supplemented with 10% fetal calf serum, were prepared into single cell suspension and inoculated in 96-well culture plates at a density of 5×10^3 cells/mL. Following 24 h routine culturing, culture medium was exchanged with CBBR 1640 medium to culture for another 24 h, with the final CBBR concentration being 0.25, 0.50, 1.00, 2.00 and 4.00 mg·mL^{-1} respectively and equivalent to such quantities of crud drugs, with 5 paralleled wells for each concentration and a blank control in all these tests. Then, medium was displaced with 150 μL 0.5% MTT solution for a culturing period of 4 h. Followed were the steps to discard MTT medium and to add DMSO solution at a volume of 150 μL/well, with culture plates transferred to a shaking table for 10 min under a shaking status at 50 r/min. After this step, OD value of each well was detected on a Fully Automatic Enzyme Label Analyzer at the wave length of 490 nm for calculating cellular inhibitory rate and IC50 concentration of CBBR. All the experiments were repeated in triplicate.

Inhibitory rate was calculated based on the following equation

$$\log \mathrm{IC}_{50} = \mathrm{Xm} - \mathrm{l}\left(\mathrm{P} - \left(3 - \mathrm{Pm} - \mathrm{Pn}\right)/4\right)$$

where Xm meant lg (maximum dose), l was lg (maximum dose/adjacent dosage), P was the sum of positive reaction, Pm was the maximum positive reaction, and Pn was the minimum positive reaction.

IC50 was calculated from the followed formula as

$$\text{Inhibitory rate}\% = \left(\mathrm{ODc} - \mathrm{ODe}\right)/\mathrm{ODc} \times 100\%$$

where OD was the abbreviation of optical density, ODc was the OD value of control group, ODe was the OD value of experimental group.

3) Autophagy-inducing effect of CBBR on CNE2 cells supplemented with 3-MA blocking test.

a) Autophagy-inducing effect determination CNE2 cells were inoculated in 6-well culture plates with a density of 1×10^5 cells/well, cultured for 24 h in 5% CO_2 incubator at 37˚C with saturated humidity. Then, cells at exponential growth phase in this culturing system were designated into three interfering groups with 3 different CBBR concentrations, *i.e.* 0.25, 0.50, 1.00 mg·mL^{-1} selected on the basis of its IC50 concentration, *i.e.* the concentrations at IC50, less than doubled the IC50 and twice the IC50 respectively. By the time of medium exchanging, all wells in interfering groups were added with CBBR medium at their corresponding concentrations in a total value of 2 mL in each well for a continuing culture period of 24 h, with a paralleled blank control group set as well. By the end of interfering culture period, medium in all culture wells was discarded. Following 2 times washing with PBS, added was the solution of 50 μM MDC into each well for a further culture of 1 h. Then, plates were observed un-

der an inverted fluorescence microscope to determine the autophagy inducing levels among different CBBR concentrations on CNE2 cells after the medium in each well being discarded and washed for 3 times with PBS. The experiment was also repeated in triplicate.

b) Blocking test on autophagy-inducing effect of CBBR Based on the determination of autophagy-inducing response as tested above, cells at exponential growth phase were inoculated into 6-well plates at a density of 1×10^5 cells/well for a culturing period of 24 h in 5% CO_2 incubator at 37˚C with saturated humidity, with four groups designated, *i.e.* blank control group (BG), single 3-MA treating group (MG), single CBBR treating group (CG) and 3-MA pretreating followed by CBBR treating group (CMG) respectively. By the time of medium exchanging, cells in the wells of MG and CMG were pretreated with 2 mM 3-MA for 1h at first. Then, exchanged were CBBR medium at IC50 concentration into the wells of CG and CMG, and normal medium into the wells of BG and MG, following a continued culturing of 24 h respectively. After that time, followed was medium exchange with 50 μM MDC adding into all wells of each group for a further culturing period of 1 h. Then, medium in all wells was discarded and cells were washed 4 times for observation and image taking under an inverted fluorescence microscope to determine the blocking effect of 3-MA on autophagy-inducing level of CBBR in a comparative way. Also, this experiment was repeated in triplicate.

c) Measurement of the positive rate of stained cells For measurement of the positive rate of fluorescein stained cells, a histological score (H-score) was formulated as the sum of the percentage of positively stained cells multiplied by the weighted intensity of staining, observed under the microscope with the magnification at 10 × 20 and positive cells numbered in 5 visual field with typical staining cells in each section. The H-score was calculated with the equation of $\sum Pi(I + 1)$, where I represented the intensity of staining, *i.e.* 0 being none, mild being 1, moderate being 2, and intense being 3, and Pi represented the fraction of stained cells for each staining intensity [25].

4) Western blot assay on the activity levels of key molecules in P13K/AKT/mTOR signaling pathway of CNE2 cells.

a) The effect of CBBR on the expressive activity of key messenger molecules in the P13K/AKT/mTOR signaling pathway of CNE2 cells.

i) Preparation of cell lysis solution. CNE2 cells were inoculated in 100 mm culture dishes at a density of 7×10^5 cells/mL, cultured in a 5% CO_2 incubator at 37˚C with saturated humidity till cells growing to an exponential growth phase. By this time, cells were designated into 3 interfering groups to be treated with the concentrations of CBBR at 0.25, 0.50, and 1.00 $mg \cdot mL^{-1}$, and added with CBBR medium at corresponding concentrations respectively and with a paralleled blank control group designated as well. Then, cells were continuously cultured for 24 h. After that time, medium in each well was discarded, cells were collected from each group routinely in an ice box for 15 min, supplemented with

50 μM RIPA lysis solution containing 1% PMSF to produce cell lysis, and followed by cells washed 3 times with cold PBS respectively. After this step, cell lysis solutions were transferred into 1.5 mL PE tubes for centrifugation at 4˚C and 12,000 rpm for 10 min, with the supernatants collected respectively by the end of this operation for following procedures, also repeated in triplicate.

ii) Determination of protein concentration. This step of operation was carried out following the instruction of operating manual of BCA protein quantitative detection kit, with BCA working solution and standard diluent prepared to draw a standard curve. Following the sample of each group loaded, they were detected on a microplate reader at 570 nm to calculate the protein concentration of each sample respectively by referring to the standard curve.

iii) Preparation of electrophoresis gels. SDS-PAGE electrophoresis gels were prepared according to the routine standard operation program.

iv) Electrophoresis. A total of 25 μL sampling volume was loaded into one well of chamber for each sample. Then, electrophoresis was carried out at a voltage of 80 V with a maximal current. When target proteins swimming to the distance larger than 1cm to the lower edge, the electrophoresis was ended.

v) Protein transmembrane. Once gels were taken out of the electrophoresis groove, they were appressed closely to the PVDF membrane and transferred into a diaphragm groove for 60 min at a 300 mA constant current.

vi) Antibody incubation. PVDF membranes were transferred into an incubator, once transmembrane ended properly, with the first antibodies added and incubated overnight. Then, the solutions of first antibody were discarded and membranes were washed 4 times with TBST, each for 5 min. After this, the second antibodies were added onto the membranes for 1 h incubation under room temperature. At last, solutions of the second antibody were discarded and membranes were washed 4 times with TBST, each time for 5 min as well.

vii) Development and image analysis. Membranes were put into a chemiluminescence gel imaging system with 0.5 - 1.0 mL developer added for development. Then, membranes were transferred to an image analyzer for image analysis with Image J software.

viii) Tested indexes were Beclin 1, LC3I, LC3II, and the ratio of LC3II to LC3I, and the key molecules in the signaling pathway for autophagy as PI3K, p-AKT, AKT and p-mTOR, with the same procedure in a paralleled comparative way to analyze the expressive level of each index.

b) Blocking tests on the effect of CBBR on the expressive activities of the key molecules in the signaling pathway of PI3K/AKT/mTOR in CNE2 cells As the same as above in (2) of 1.2.3, the blocking test on autophagy-inducing effect of CBBR, CNE2 cells were inoculated into the 100mm culturing dishes at the density of 7×10^5 cells/mL and incubated in 5% CO_2 incubator at 37˚C for 24 h. Then, culturing dishes, when cells getting into the exponential growth phase, were designated into four groups, *i.e.* blank group (BG), 3-MA treating group (MG), CBBR treating group (CG) and CBBR plus 3-MA pretreating group

(CMG) respectively. By the time of medium exchanging, cells in the dishes of MG and CMG were pretreated with 2 mM 3-MA for 1h at first, and then, exchanged were CBBR medium at IC50 concentration into the dishes of CG and CMG and normal medium into the dishes of BG and MG for a continued culturing of 24 h respectively. After this step, followed were cell collection, cell lysis solution preparation, determination of protein concentration, electrophoresis, protein transmembrane, antibody incubation, development and image analysis, and the determination on the expressive levels of the key molecules in the signaling pathway of PI3K/AKT/mTOR in these cells respectively, as the same in (1) of 1.2.4. Also, this series of experiment was repeated in triplicate.

2.3. Statistical Processing

SPSS21.0 statistical software (IBM, Chicago, IL, USA) was utilized to the analysis of these experimental data. One-way ANOVA and Dunnett's multiple comparisons test or Tukey's multiple comparisons test were performed in this process for various cases respectively. Results were reported as mean ± SD. Data were considered significant statistically when $P \leq 0.05$.

3. Results

3.1. The Inhibitory Effect of CBBR on the Proliferating Activity of CNE2 Cells

Following 24 h incubation with CBBR, cells showed various levels of proliferation activity inhibition responsible for different concentrations of CBBR as shown in Figure 1. When incubated with CBBR at the concentration of 0.25

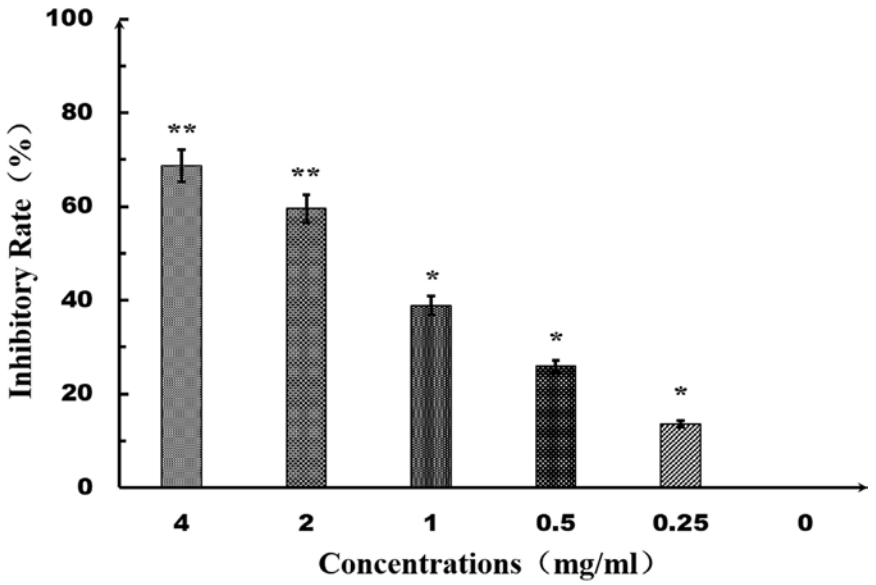

Figure 1. The inhibitory effect of CBBR on the proliferation activity of CNE2 cells indicated as inhibitory rate (%) as determined by MTT assay for IC50 determination at different concentrations (4.00, 2.00, 1.00, 0.50, 0.25, 0.00 mg/mL respectively). *($P < 0.05$), **($P < 0.01$).

mg·mL^{-1}, CNE2 cells displayed a surviving rate of 86.54% ± 1.21%, when CBBR at the concentration of 0.50 mg·mL^{-1}, the surviving rate was 73.74% ± 2.60%, at the concentration of 1.00 mg·mL^{-1}, the surviving rate was 59.24% ± 2.24%, at the concentration of 2.00 mg·mL^{-1}, the surviving rate was 41.1% ± 4.46%, and at the concentration of 4.0 mg·mL^{-1}, the surviving rate significantly decreased to the level of 29.98% ± 1.31% ($P \leq 0.01$), obviously showing a concentration-dependent mode. It was suggested from these results that CBBR held very strong inhibitory effect on the proliferation activity of CNE2 cells. In order to explore the strength of autophagy-inducing effect of CBBR at various concentrations on CNE2 cells properly, such levels of concentration of CBBR, *i.e.* 0.25 mg·mL^{-1}, 0.50 mg·mL^{-1} and 1.00 mg·mL^{-1}, around its IC50 concentration of 0.50 mg·mL^{-1}, were selected as the intervening concentrations to target cells in the following experiments (see Figure 1).

3.2. Autophagy-Inducing Effect of CBBR on CNE2 Cells

It was shown that the number of autophagosomes increased gradually with correspondingly increased MDC fluorescent brightness in these cells following the elevation of CBBR intervening concentrations treated for 24 h as shown in Figure 2 and Figure 3 ($P < 0.01$). The maximal number of autophagosomes was seen in the group of cells treated by 0.50 mg·mL^{-1} CBBR, with the strongest fluorescent brightness here. On the contrary, the number of autophagosomes declined in the group of cells treated by the concentration of 1.00 mg·mL^{-1} CBBR, when compared with that of the former group, was just slightly higher than that of cells treated by 0.25 mg·mL^{-1} CBBR. These results certainly confirmed that CBBR held obvious autophagy-inducing effect on CNE2 cells, while the strength of this kind of effect did not completely parallel to its concentration levels, *i.e.* being not a proportional relation between the applied concentrations and the effect potencies (see Figure 2 and Figure 3). Once pretreated by autophagy-blocking reagent 3-MA in the culturing system, displayed was still relatively stronger inducing effect on autophagic activity as compared with that of blank

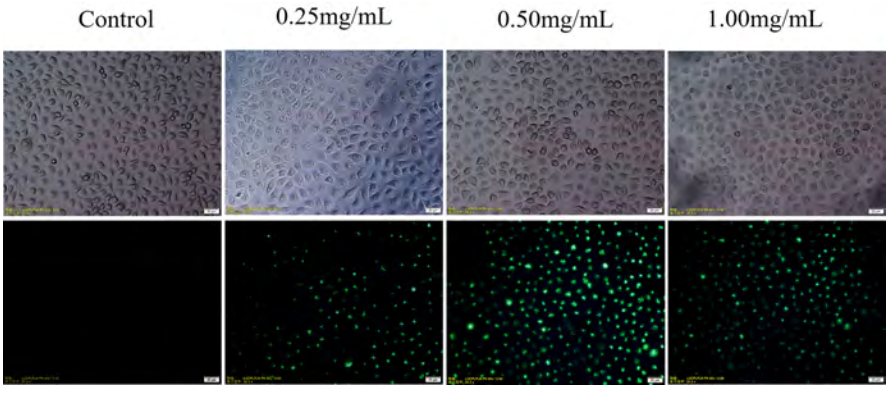

Figure 2. Autophagic activity of CNE2 cells induced by CBBR at different concentrations (1.00, 0.50, 0.25, 0 mg/mL respectively) as displayed by MDC fluorescent labelling images (100×).

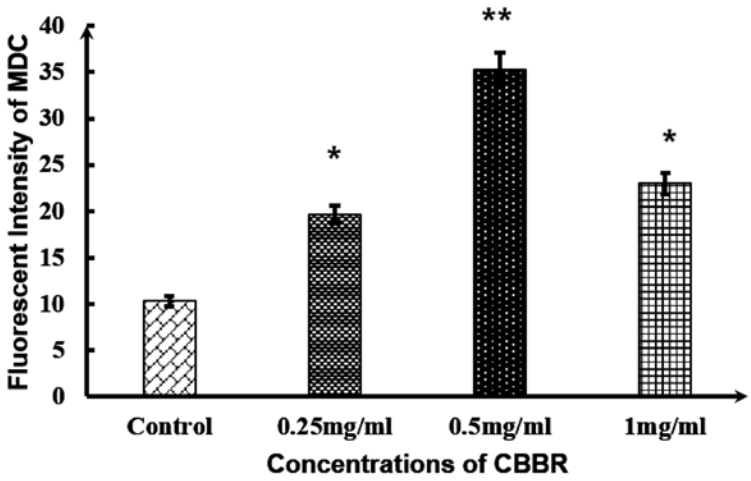

Figure 3. Comparison on the autophagy-inducing effect of CBBR at different concentrations on CNE2 cells as assayed by MDC fluorescent labelling images with the results displayed in histogram. *($P < 0.05$), **($P < 0.01$).

controlling group and negative controlling group just without 3-MA treated, while the active strength of autophagy-inducing effect was shown some degrees of decrease when compared with that without 3-MA treatment. These data meant that the autophagy-inducing effect of CBBR could not be completely blocked by 3-MA. Moreover, it could be found further that cells in the group pretreated by 3-MA and following 0.5 mg/mL CBBR treatment still showed a significantly stronger autophagy activity when compared with that in the group of cells simply treated by 3-MA alone, though its autophagy-inducing effect declined at some level, even being not at an obviously blocked status as shown in **Figure 4** and **Figure 5**. All these suggested that the autophagy-inducing effect of CBBR on CNE2 cells could not be completely blocked by 3-MA, showing a kind of different effective mechanism at some levels with that of classic pathway, perhaps another unknown pathway(s) for such a phenomenon present here.

3.3. The Effect of CBBR on the Expressive Activity of Autophagy-Specific Proteins of CNE2 Cells

Beclin-1 and LC3 are the specific protein markers of autophagy, showing a positive proportional relation with their expressive activities as well as the ratio of LC3II to LC3I with the autophagic level. Especially, there was a similar proportional increase in the protein fragment of LC3II as shown in the electrophoresis strip and the level of autophagy. Therefore, the level of LC3II:LC3I ratio can be taken as the key index to estimate the strength of autophagic activity. As shown in **Figure 6**, the expression of Beclin-1 and LC3II were significantly elevated ($P <$ 0.05 - 0.01) in CNE2 cells following the treatment of CBBR for 24 h at various concentrations as shown in **Figure 6**, with the maximal expressive levels being Beclin-1 and LC3II proteins as well as the highest peak value of LC3II:LC3I ratio about 5.1 in the group of cells treated by the concentration of 0.50 mg·mL^{-1} CBBR ($P < 0.01$). Furthermore, the rank of change in the size of ratio was in

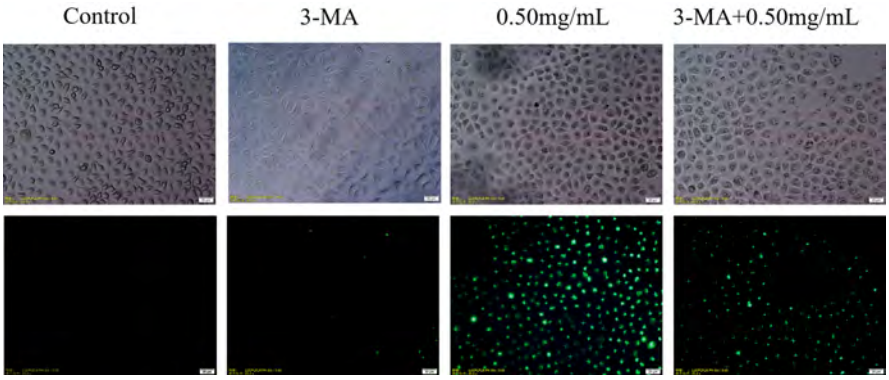

Figure 4. Blocking test comparison with 3-MA on the autophagy-inducing effect of CBBR at IC50 concentration on CNE-2 cells as displayed by MDC fluorescent labelling images (100×).

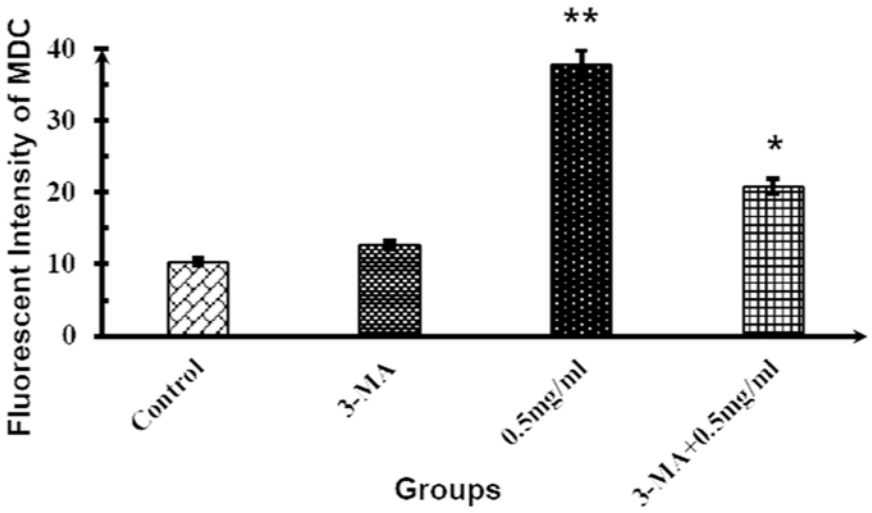

Figure 5. Comparison on the autophagic-inducing effect of CBBR at IC50 concentration combined with blocking test by 3-MA on CNE2 cells assayed by MDC fluorescent labelling images with the results displayed in histogram. *($P < 0.05$), **($P < 0.01$).

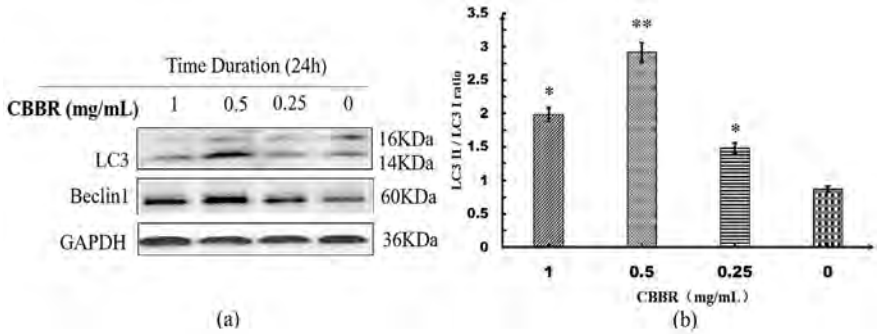

Figure 6. The effect of various concentrations of CBBR (1.00, 0.50, 0.25, 0.00 mg·mL^{-1} respectively) on the expressive levels of autophagy-specific proteins LC3II and LC3I of CNE2 cells. (a) The electrophoretogram of Western blot assay on LC3 and Beclin 1 following intervention with various concentrations of CBBR; (b) Comparison on the effect of various concentrations of CBBR on the expressive level ratios of autophagy-specific proteins LC3II to LC3I with the results displayed in histogram. *($P < 0.05$), **($P < 0.01$).

such an order, with the maximal magnitude being the cells treated by the concentration of 0.50 mg·mL^{-1} CBBR, then being those treated by CBBR at the concentrations at 1.00 mg·mL^{-1}, followed by 0.25 mg·mL^{-1}, and 0 mg·mL^{-1} respectively from the highest to the lowest. When the group of cells treated by the concentration of 0.50 mg·mL^{-1} was taken as the base line, it was shown that the ratio changes of LC3II:LC3I caused by the changes in the concentrations of CBBR, either elevated or declined, did not completely parallel to the changes in the levels of autophagic activity, instead, changes in the concentrations of CBBR around 0.50 mg·mL^{-1} induced decreasing in LC3II:LC3I ratios and autophagic activities, see Figure 6. It might be suggested here that there should be an optimal concentration level present for CBBR effect on the target cells to induce the highest autophagic response. This would be somewhat different with the characteristics in morphological changes of autophagy as marked by fluorescein labeling.

It was shown in the blocking test that the expressive levels of Beclin-1 and LC3 II proteins in the group of cells pretreated with 3-MA followed by CBBR treatment at the concentration of 0.50 mg·mL^{-1} for 24 h were still obviously higher than that of the negative controlling group untreated with CBBR, though showing a significantly decreased tendency when compared with that of cells simply treated with 0.50 mg·mL^{-1} CBBR but not pretreated with 3-MA. Furthermore, it was also displayed that 3-MA could only partially inhibit the autophagy-inducing activity caused by CBBR, while it could not completely block such an autophagy-inducing effect of CBBR on target cells as shown in Figure 7. Among these groups of blocking experiment, the strength of autophagy-inducing effect, as indicated by the ratio of LC3II:LC3I, was ranked in this order of CBBR intervening group not treated by 3-MA, CBBR intervening group pretreated by 3MA, the group simply treated by 3-MA, and the blank controlling group from the higher to the lower, with no significantly statistical significance

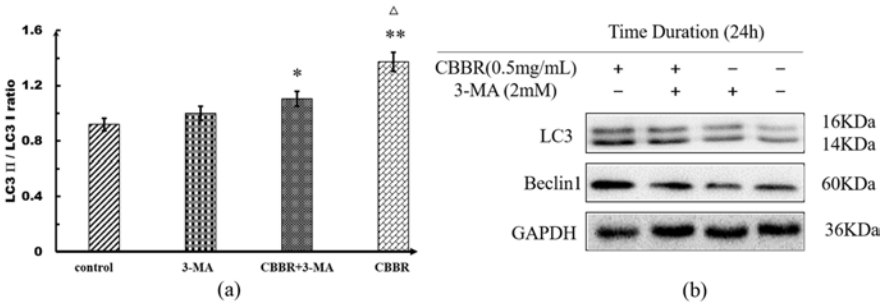

Figure 7. The blocking effect of 3-MA on the expressive levels of autophagy-specific proteins LC3II, LC3I, their ratio and Beclin 1 in CNE2 cells induced by CBBR at IC50 concentration (0.5 mg·mL^{-1}). (a) Comparison on LC3II/LC3I ratios in the 3-MA blocking test with the results displayed in histogram; (b) The electrophoretogram of Western blot assay on LC3 and Beclin 1 tested in 3-MA blocking test. *($P < 0.05$), **($P < 0.01$), indicating the statistical significance when compared with the data of controlling group, and $^{\Delta}$($P < 0.05$), indicating the statistical significance when compared with the data of the group treated by CBBR+3-MA.

present between the last two groups ($P > 0.05$), while the most prominent strength of autophagy-inducing effect was determined in the group of cells intervened with CBBR alone but not treated by 3-MA, significantly higher than that of all the other groups. Although the inducing effect of the group pretreated by 3-MA followed by CBBR treating (CMG) was obviously lower than that of the former, it was still higher than that of simply treated by 3-MA, see (Figure 7). These data suggested that 3-MA could not completely block the autophagy-inducing effect of CBBR on CNE2 cells again, and CBBR might bring about autophagy response in CNE2 cells via other pathways in part.

3.4. The Effect of CBBR on the Activities and Phosphorylation Levels of the Key Messenger Molecules in PI3K/AKT/mTOR Signaling Pathway of CNE2 Cells

Treatment with different concentrations of CBBR could bring about somewhat different characteristic effect on the activities of the key messenger molecules in PI3K/AKT/mTOR signaling pathway of CNE2 cells. Taking the internal reference as the baseline to analyze the corresponding changes in the activities and their phosphorylation levels of the key molecules in PI3K/AKT/mTOR signaling pathway of the cells treated by CBBR for 24 h, it was shown, when compared with that of controls, all groups of cells intervened by CBBR showed a down-regulated tendency in the activity levels of PI3K, AKT, p-AKT and p-mTOR ($P < 0.05$ or $P < 0.01$), indicating that significantly declined were the expressive and/or phosphorylation levels of the key molecules in this signaling pathway, with an obviously responsible feature showing a concentration-dependent manner for PI3K and p-AKT to CBBR intervening, *i.e.* the effect variation ratio being maximal at the highest concentration of 1.00 $mg{\cdot}mL^{-1}$ CBBR, followed by a decreased changing tendency in the effect variation ratio following the decline of CBBR concentration. However, the features of change in the response of AKT and p-mTOR showed a super-optimal concentration manner, *i.e.* their activity levels reached at the maximum at the concentration of 0.50 $mg{\cdot}mL^{-1}$, almost similar with that of specific autophagic index LC3II:LC3I ratio response determined by Western blot assay. These data might suggest that there should be possible for different messenger molecules in the same signaling pathway varying in their response manner to a same intervening factor. Therefore, it should be possible that there might be other no classic signaling pathways present for autophagy inducing besides that could be blocked by 3-MA, as seen in Figure 8 and Table 1.

4. Discussion

It has been confirmed widely that berberine holds very strong antitumor effect for long time via such effects as inhibiting cell proliferation activity, blocking cell cycle at G2 phase, inhibitory effect on the synthesis of DNA and protein, suppressing the biological synthesis response through integrating TOPI at S phase of the cell cycle, inducing apoptosis, repressing the potency of migration and

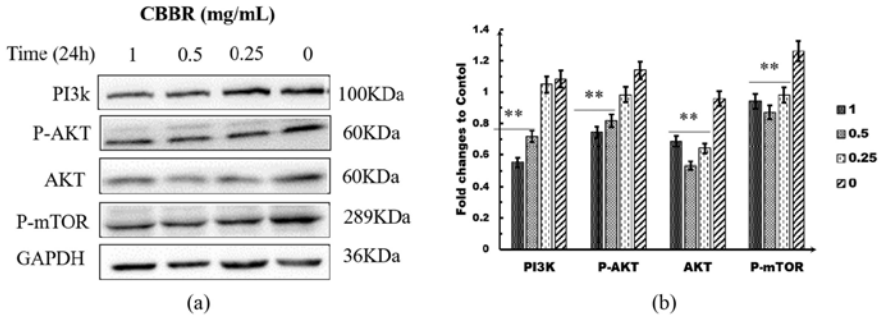

Figure 8. Comparison on the expressive levels and changeable characteristics of key messenger molecules in P13K/AKT/mTOR signaling pathway during the period of autophagy induced by various concentrations of CBBR (mg·mL^{-1}). (a) The electrophoretogram of Western blot assay on the key messenger molecules in P13K/AKT/mTOR signaling pathway during the period of autophagy induced by various concentrations of CBBR; (b) Comparison on the fold ratios of various key messenger elements responsible for autophagy in P13K/AKT/mTOR signaling pathway displayed in histogram. *($P < 0.05$), **($P < 0.01$).

Table 1. Effects of CBBR at various concentrations (mg·mL^{-1}) on the key molecule messenger in PI3K/AKT/mTOR signaling pathway measured by image analyzer.

CCBR concentrations	0.00	0.25	0.50	1.00	P
PI3K	1.10	1.08	0.73	0.58	0.016
p-AKT	1.15	0.98	0.83	0.75	0.003
AKT	0.92	0.63	0.53	0.71	0.000
p-mTOR	1.30	1.00	0.88	0.96	0.003

metastasis, and inducing differentiation through decreasing the expression of c-k-ras gene of tumor cells, and improving the status of immune function and dyscrasia of host with tumor. The main active component of CBBR is berberine, meaning that it should hold such an antitumor effect as well, as previously confirmed in our laboratory that EAE, the ethyl acetate extract from c compound *Coptischinensi* formula, can promote peripheral blood DCs of patients with NPC to mature differentiation to elevate their potentiality in antigen presenting and to activate T cell mediated cellular immune function [24]. The present work is mainly focused on the exploration of autophagy-inducing effect brought about by CBBR on CNE2 cells and its main mechanism of this kind of effect.

There was a different responsiveness present for CBBR between its cell proliferation inhibitory effect and the autophagy-inducing effect as shown in this work, a plateau-like feature taking place in the autophagic activity of CNE2 cells following the intervening with CBBR, while the inhibiting effect on cellular proliferation shown a nearly linear concentration-dependent way and keeping a paralleled tendency all along with the concentration level changed. The level of autophagic response induced by CBBR above the concentration of IC50 was not completely in a proportional way to the increased concentration, since no autophagy displayed when treated without CBBR as in blank control group, being

minimal when treated by 0.25 mg·mL^{-1}, and being maximal when treated by 0.50 mg·mL^{-1}, but only a little stronger autophagy occurred than the activity level as seen in the group of 0.50 mg·mL^{-1} when treated by 1.00 mg·mL^{-1}. These data suggested that there was a plateau-like phenomenon with a peak value present in the autophagy-inducing effect of CBBR on CNE2 cells. Perhaps such an effect of CBBR was only one of its mechanisms against NPC cells. This fact should be more in line with the characteristics of CBBR in its actual pharmacological effect.

Blocking test could provide still more reliable information to explain many complex results in the research of biological mechanisms as found in this work. Therefore, autophagy-blocking tests were set up here. As also seen here, there was no autophagic response found in the blank controlling group, and occasionally found autophagic cells present in the group of cells treated by 3-MA alone. However, there was a very strong autophagic response present in the group of cells treated simply by CBBR at the concentration of 0.50 mg·mL^{-1}, while it became weaker at some degrees in the group of cells pretreated with 3-MA followed by the treatment of the same CBBR concentration, but still obviously stronger than that in the group of cells treated only by 3-MA. So, it could be concluded that 3-MA might block the autophagy-inducing effect of CBBR on CNE2 cells at some levels, but it should be impossible to completely block such an inducing effect. This should suggest that CBBR might induce autophagy bypassing, at least in part, its classic, or conventional, pathway to give rising a much stronger autophagy-inducing effect on target cells. Still more, there may be other much complex multiple signaling pathways involved in such a complicated phenomenon.

The level of autophagy-inducing effect could be evaluated most effectively by its very specific indexes such as Beclin 1 and LC3II:LC3I ratio. So, these indicators were taken to evaluate the inducing effect of CBBR on autophagy. As shown from the results of Western blot, different concentrations of CBBR caused a consistent mode of inducing effect on target cells in the response of autophagy, no matter Beclin 1 (60 kDa), LC3I (16 kDa), LC3II (14 kDa), or LC3II:LC3I ratio. Here, the response strength of autophagic indicators to CBBR were ranked in the order from the highest to the lowest as the group of 0.50 mg·mL^{-1}, the group of 1.00 mg·mL^{-1}, the one of 0.25 mg·mL^{-1} and that of 0 mg·mL^{-1}. Here, it confirmed that the maximal effective concentration level of CBBR and its effect mode on CNE2 cells were almost the same as stated above once more. Since up-regulation of Beclin 1 expression could promote autophagy taking place to bring about the effect of tumor suppressor, it should be clear that significantly elevated level of Beclin 1 accompanied with very strong autophagic activity in CNE2 cells would certainly indicate that CBBR induced marked elevation of autophagy was brought about by activating the expression of Beclin 1.

Meanwhile, the ratio level of LC3-II:LC3-I can be taken as the specific index to evaluate the trend in the intensity of autophagy since the change of LC3-II in

its expressive level was paralleled to the forming rate of sub-cellular autophagic structures. As shown in the interfering test with CBBR and blocking test with 3-MA, the expressive levels of LC-3 were both significantly elevated, with corresponding LC3-II/LC3-I ratio increased obviously as well. When taken such a ratio as the indicator to evaluate the autophagic activity in these experiments, the intensities of autophagic response could be ranked in such an order from the higher to the lower as the group of cells interfered with CBBR alone, the group of cells interfered with CBBR plus 3-MA pretreatment, and the blank group. This trend in the intensity change of autophagic response was extremely similar with that reflected by the morphological indicators of autophagy from the results of autophagy-blocking test, all showed a phenomenon suggesting that autophagy blocker 3-MA could not completely block the autophagy-inducing effect of CBBR on NPC cells. Once again, it should be possible that there would be other nonclassical pathway(s) present for CBBR to induce intensive autophagic response in NPC cells other than the classic one. It should be worth exploring in future.

The mechanism of autophagy for CNE2 cells might involve in the signaling pathway of PI3K-Akt-mTOR and their phosphorylation levels. As shown in **Table 1**, these cells displayed obviously elevated expressive levels of PI3K and p-AKT in a concentration-dependent mode, increasing with the elevation of CBBR concentration to the highest peaks of value at 1.00 $mg{\cdot}mL^{-1}$, while the peak expressive levels of AKT and p-mTOR were seen at such a CBBR concentration of 0.50 $mg{\cdot}mL^{-1}$. These effective features indicated that they were kept in a change trend similar with that of specific autophagic proteins, meaning that this pathway should be involved in the initiation of autophagy and its speeding up in signaling transduction.

However, there were some other special features shown from these experiments for the effect mode of CBBR on mTOR. As shown from the relative ratio of background activity of mTOR, the value at this level was 0.96, while it reduced to 0.88 at lower CBBR concentration of 0.25 $mg{\cdot}mL^{-1}$ showing an inhibitory effect significantly lower than its background activity. However, it elevated obviously when the concentration of CBBR increased to 0.50 $mg{\cdot}mL^{-1}$ and then, got to a peak value of 1.30 at the concentration of 1.00 $mg{\cdot}mL^{-1}$, also appeared a similar trend in the change of activity with that of PI3K [26]. Evidently, this phenomenon was closely associated with the effect of mTOR as a negative regulatory factor on autophagic signaling pathway, involved in the initiation of mTOR biosynthesis to inhibit autophagy, or it could initiate autophagy [27]. Such a special reactive mode of mTOR to the interfering of CBBR further meant that there would be other possible underlying mechanism present and was also consistent with the finding of the autophagy caused by 11-MT that was found to be brought about via activation of the AMP activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) and the c-Jun N-terminal kinase (JNK) signaling pathways [28].

Based on the discussion above, we could deduce that one of the important

mechanisms for CBBR to inhibit the proliferating activity of CNE2 cells might be achieved through activating their autophagic response, as that of quercetin to promote hepatoma cells going into apoptotic program via inducing autophagy at first [29]. Meanwhile, curcumin could induce apoptosis in malignant mesothelioma cells through initiating autophagy flow so as to suppress their level of proliferation [30]. Some ingredients of herbal medicine, such as TanshinoneI (TSI) and Aspidosperma alkaloids, held anticancer potential against human glioma U87 MG cells and could reverse the resistance of chemical drugs against cells of ovarian cancer via inducing apoptosis and autophagy through ER stress and AKT signaling induced by TS I via intracellular reactive oxygen species accumulation [28] [31], though some reports indicated that autophagy was significantly associated with tumor grade and EMT, conferring the survival advantage to neoplastic cells to anti-cancer therapies and significantly affecting the invasive potential of cancer cells and supports their metastatic dissemination in a tissue and tumor stage dependent manner [32] [33]. However, it has been confirmed recently that PHLPP2 was able to mediate BECN1/Beclin1 stabilization indirectly to promote BECN1-dependent macroautophagy/autophagy to inhibit BC tumor cell growth and that increased autophagy via attenuating *MIR*516*A* resulted in a dramatic inhibition of xenograft tumor formation *in vivo* [34].

5. Conclusion

In summary, one of the main effective characteristics of CBBR on NPC cells should be prominently the autophagy-inducing effect, one of the important mechanisms for CBBR to inhibit, even to kill, tumor cells through which the proliferating activity of CNE2 cells can be inhibited significantly, even though autophagic response could induce various, even contrary, effects on different kinds of tumor cells [35] [36].

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Non-Alcoholic Fatty Liver Disease as a Coronary Heart Disease Severity Predictor

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Abstract

Background and Aim: Association of non-alcoholic fatty liver disease (NAFLD) and liver related mortality is well-known; however; its cardiovascular risk remains questionable. The aim of this work was to highlight the association between NAFLD and the severity of coronary heart disease. **Method:** The study was conducted between July and December 2019 at Qena university hospital, Qena, Egypt on patients who were scheduled for coronary angiography. Liver steatosis was diagnosed using FibroScan and the severity of coronary artery occlusion was assessed by Gensini score calculation. Statistical analysis was done to study the correlation between liver steatosis and angiography findings. **Result:** A total of 100 patients were included, mean age 48 years (±8 SD). Correlation between coronary angiography and the baseline variables revealed statistically significant positive correlation between Gensini score and ALT ($p = 0.002$), FBS ($p < 0.001$), steatosis ($p < 0.001$) and diabetes ($p < 0.001$). Also, gradual increase in the Gensini score with higher steatosis grades was noticed, medians: 10.5, 21.7, 57.6 and 102.6 in S0, S1, S2 and S3 respectively, $p = 0.01$. Variables that have significantly predicted higher Gensini score in univariable regression analysis were: diabetes (OR: 55.6, CI: (41 - 70)), hypertension (OR: 19, CI: (2 - 37)), F2-F4 fibrosis (OR: 33, CI: (24 - 38)) and S2-S3 steatosis (OR: 34, (23 - 40)), while in multivariable regression; the statistically significant variables were: diabetes (OR: 23, CI: (10 - 37)), hypertension (OR: 11, CI: (4.8 - 22)) and S2-S3 steatosis (OR: 24, CI: (17 - 31)). **Conclusion:** NAFLD is an independent cardiovascular risk predictor with statistically significant increase in Gensini score with the higher grades of NAFLD.

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Keywords

NAFLD, FibroScan, Gensini Score

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a worldwide health problem with an increasing prevalence reaching about 75% of chronic liver disease (CLD) in the developed countries, which makes it the most common CLD in the western world [1]. In a recent meta-analysis, the global prevalence of NAFLD was estimated to be around 25% with highest prevalence in the Middle East and South America and lowest in Africa [2].

Traditionally, NAFLD has linked to liver-related morbidities through its progression to non-alcoholic steatohepatitis (NASH) with subsequent liver cirrhosis, and it is expected that NAFLD related liver diseases will be the leading cause of liver transplantation by 2030 [3]. In addition to this obvious link between NAFLD and end-stage liver disease, several studies suggested the presence of an association between NAFLD and cardiovascular disease (CVD). Recently, a large body of evidence has supported this suggestion and classified CVD as the main cause of death in patients with NAFLD [4] and considered NAFLD as a significant independent risk factor for subclinical and clinical CVD in the absence of the classic cardiovascular risk factors [5] [6].

In contrary to the traditional cardiovascular risk factors like diabetes and obesity, and despite the growing evidence for the association of CVD with NAFLD, the exact mechanism of this association and the degree of correlation between the grade of NAFLD and the severity of CVD are not well-studied [7] [8] [9]. The aim of our work was to study the correlation between NAFLD and the severity of coronary heart disease (CHD) as determined by Gensini score.

2. Materials and Method

2.1. Patients

Patients without prior history of definite ischemic event who were scheduled for coronary angiography from July 2019 - December 2019 at Qena university hospital, Qena, Egypt were included, while those with known history of definite CVD or heart failure were initially excluded. Patients with prior history of CLD including chronic hepatitis C (CHC), chronic hepatitis B (CHB) and NAFLD/NASH or renal impairment were excluded. After initial abdominal ultrasound (US), patients with definite cirrhotic echo pattern or any confounding factor interferes with accurate transient elastography (TE) reading such as hepatocellular carcinoma, ascites or morbid obesity were excluded.

2.2. Transient Elastography (TE)

Liver stiffness measurement (LSM) and controlled attenuation parameter (CAP)

were obtained by an expert operator using FibroScan® device (Echosense, Paris, France). The procedure was performed after 8-hour fasting while the patient in the supine position. Result was not considered reliable except after acquisition of 12 successful readings with interquartile range/median ratio less than 30% [10]. LSM was used to estimate the METAVIR fibrosis stage as follows: F0-F1: 2.5 - 6.9 kPa; F2: 7.0 - 9.4 kPa; F3: 9.5 - 12.4 kPa; F4: ≥ 12.5 kPa [11]. CAP was expressed in dB/m and its values were used to estimate steatosis stage as follows: S0 < 238 dB/m, S1: 238 - 258 dB/m, S2: 259 - 291 dB/m and S3: ≥ 292 dB/m [12].

2.3. Coronary Angiography

Coronary angiography was performed within 4 weeks of enrollment by an expert cardiologist blinded about the LSM & CAP readings of the patients. Gensini score was then calculated as mentioned in the literature [13] [14].

2.4. The Studied Variables

Gensini score will be correlated to the continuous variables including: age, fasting blood sugar (FBS), total serum cholesterol (TC), triglycerides, high density lipoprotein (HDL), alanine transaminase (ALT), aspartate transaminase (AST) and serum bilirubin, and categorical variables including: gender, diabetes mellitus (DM), hypertension (HTN), smoking, liver fibrosis (F0-F4) and liver steatosis (S0-S3).

2.5. Statistical Analysis

Categorical variables are expressed as number and percent, continuous variables as median and interquartile ranges (IQR). Chi squared test was used to compare non-parametric variables. Pearson correlation was used to study the correlation between Gensini score and other variables. Univariate and multivariate logistic regression was calculated to identify the statistically significant CVD risk predictors. Analysis was performed using SPSS® version 22. $p < 0.05$ was considered statistically significant.

2.6. Ethical Clearance

The study protocol was concomitant with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethical committee of Qena Faculty of Medicine, South Valley University, Qena, Egypt. A written informed consent was obtained from all included patients before enrollment in this study.

3. Result

A total of 104 patients were initially enrolled in this work, 4 patients were excluded from our analysis; 3 had abnormal renal biochemistry and 1 had microalbuminuria. The baseline criteria of the finally included 100 patients are shown in **Table 1**; the mean age was 48 years (±8 SD), 30% were females, 52% were smokers, 30% had type 2 diabetes and 36% had essential hypertension. The rest

Table 1. Baseline criteria in the studied patients. Categorical variables are expressed as number and percent while continuous variables are expressed as median and interquartile ranges (IQR 25 & IQR 75) except the age which is expressed as mean and standard deviation.

Baseline Variables		Studied Patients (n = 100)
Age (years)		47.9 ± 8.4 (Mean ± SD)
Females		30 (30%)
Smokers		52 (52%)
Hypertensive		36 (36%)
Diabetics		30 (30%)
Fatty Liver in the US		42 (42%)
Steatosis	S0	12 (12%)
	S1	38 (38%)
	S2	22 (22%)
	S3	28 (28%)
Fibrosis	F0	16 (16%)
	F1	56 (56%)
	F2	24 (24%)
	F3	2 (2%)
	F4	2 (2%)
RBS (mg/dl)		110 (100 - 150)
Total Cholesterol (mg/dl)		204.5 (162 - 237)
HDL (mg/dl)		38.5 (35 - 41)
Triglycerides (mg/dl)		179 (105 - 260)
ALT (U/L)		30 (17 - 53)
AST (U/L)		24 (17 - 35)
Bilirubin (mg/dl)		0.57 (0.4 - 0.86)

of the baseline criteria including liver fibrosis (F0-F5) & steatosis (S0-S3), lipid profile, random blood sugar (RBS), serum creatinine, alanine transaminase (ALT), aspartate transaminase (AST), serum bilirubin and serum albumin were also shown.

Correlation between the result of coronary angiography and the continuous baseline variables has revealed statistically significant positive correlation between Gensini score with ALT ($r = 0.4$, $p = 0.002$) and FBS ($r = 0.6$, $p < 0.001$), while the rest of variables showed statistically insignificant correlation, Table 2.

Correlation between Gensini score and categorical variables is shown in Table 3, in which fibrosis, steatosis and diabetes have shown statistically significant positive correlation ($p < 0.001$). The other categorical variables including hypertension, smoking and female gender had statistically insignificant correlation.

Figure 1 shows a statistically significant difference between the Gensini score in patients with early fibrosis versus those with significant fibrosis (median: 24 versus 74, $p = 0.002$), while Figure 2 shows gradual increment in the Gensini

Table 2. Correlation between Gensini score and the continuous variables.

Variables	(r)	p-value
Age (Years)	0.03	0.8
Total Cholesterol (mg/dl)	0.16	0.2
Triglycerides (mg/dl)	0.19	0.1
HDL (mg/dl)	−0.2	0.1
ALT (U/L)	0.43	0.002
AST (U/L)	0.034	0.8
Bilirubin (mg/dl)	0.19	0.1
FBS (mg/dl)	0.65	<0.001

(r): Pearson correlation coefficient.

Table 3. Correlation between Gensini score and the categorical variables.

Categorical Variables		Gensini Score	p-value
Steatosis	S0 (n = 12)	8 (5 - 16)	<0.001
	S1 (n = 38)	24 (15 - 25)	
	S2 (n = 22)	56 (32 - 68)	
	S3 (n = 28)	93 (86 - 124)	
Fibrosis	F0-F1 (n = 72)	24 (15 - 49)	<0.001
	F2-F4 (n = 28)	93 (68 - 112)	
DM (n = 30)		89 (68 - 124)	<0.001
Hypertension (n = 36)		25 (16 - 68)	0.1
Smoking (n = 52)		28.5 (15 - 68)	0.1
Females (n = 30)		32 (24 - 91)	0.08

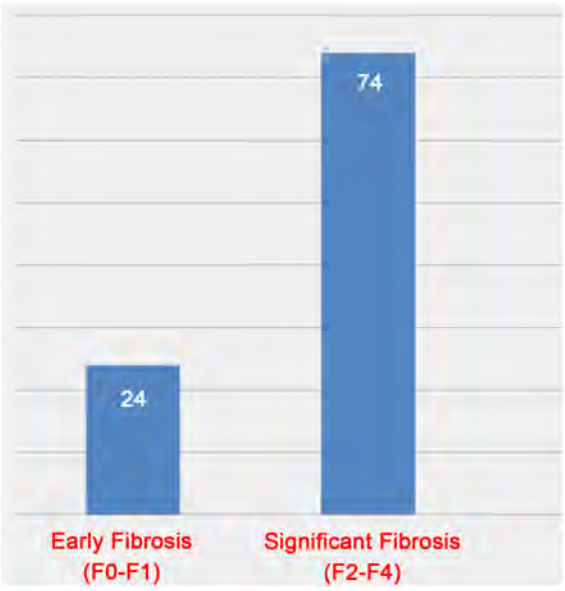

Figure 1. Difference between the median Gensini score in patients with early fibrosis (F0-F1) versus those with significant fibrosis (F2-F4), p = 0.02.

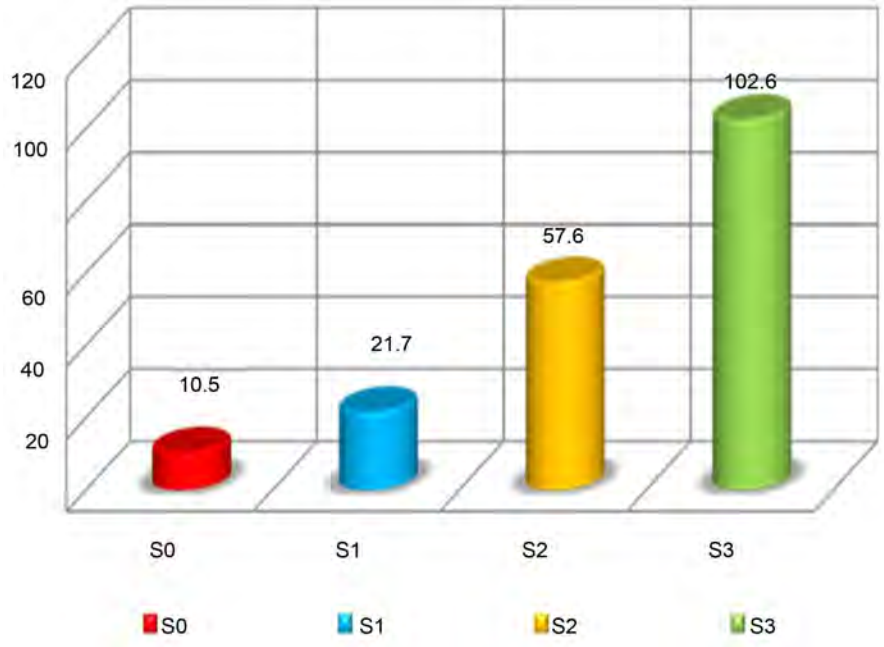

Figure 2. Difference among Gensini scores in patients with the different steatosis groups, p = 0.01.

score with the higher steatosis grades; medians: 10.5, 21.7, 57.6 and 102.6 in S0, S1, S2 and S3 respectively, p = 0.01.

Independent variables that have significantly predicted higher Gensini score in univariable regression analysis were: diabetes (OR: 55.6, 95% CI: (41 - 70), p = 0.001), hypertension (OR: 19, 95% CI: (2 - 37), p = 0.02), F2-F4 fibrosis (OR: 33, 95% CI: (24 - 38), p = 0.001) and S2-S3 steatosis (OR: 34, 95% CI: (23 - 40), p = 0.001), while in multivariable regression analysis; the statistically significant variables were: diabetes (OR: 23, 95% CI: (10 - 37), p = 0.001), hypertension (OR: 11, 95% CI: (4.8 - 22), p = 0.04) and S2-S3 steatosis (OR: 24, 95% CI: (17 - 31), p = 0.001), **Table 4**.

4. Discussion

Regarding its pathologic features, disease onset and progression, NAFLD is strongly linked to obesity, diabetes and metabolic syndrome. However; recent studies have recognized NAFLD as a separate entity independent of these diseases based on the notice that not all diabetics have NAFLD and not all NAFLD patients are obese [9] [15], also genetic predisposition to NAFLD has recently been identified in genome-wide studies [16] [17] [18] [19]. Therefore, NAFLD may play a direct role in the development and progression of CVD [20]. In the other hand; as CVD is the most common cause of death in patients with NAFLD, the 2016 European Association for the Study of the Liver (EASL) and the 2018 American Association for the Study of Liver Diseases (AASLD) guidelines recommend mandatory screening for cardiovascular health and aggressive modification of CVD risk for all NAFLD people [9] [15].

Table 4. Univariate and multivariate regression of the studied variables.

Independent Variable	Univariate		Multivariate	
	Odds Ratio	p-value	Odds Ratio	p-value
Diabetes	55.6 (41 - 70.5)	0.001	23.4 (10 - 37)	0.001
Hypertension	19.4 (2.1 - 37)	0.02	11 (4.8 - 22)	0.04
Gender	16 (3 - 34)	0.08	3.8 (0.6 - 4.8)	0.4
Cholesterol	0.2 (0.1 - 0.3)	0.1	0.04 (0.01 - 0.2)	0.5
Triglycerides	0.07 (0.002 - 0.2)	0.05	0.04 (0.01 - 0.09)	0.1
HDL	1.1 (0.4 - 2.2)	0.05	0.5 (0.2 - 1.2)	0.1
Fibrosis (F2-F4)	33 (24 - 38)	0.001	7.6 (0.7 - 15.8)	0.07
Steatosis (S2-S3)	34 (23 - 40)	0.001	24 (17 - 31)	0.001

In two cross-sectional studies by Targher *et al.* [21] [22] on 343 type 1 and 2839 type 2 diabetics, authors have concluded that NAFLD is associated with higher prevalence of CVD after adjusting for conventional CVD risk factors and metabolic syndrome components with odds ratios of 7.6 (CI: 3.6 - 24) and 1.49 (CI: 1.1 - 2) respectively. Interestingly; a meta-analysis of 34 studies reported an association between NAFLD and increased risk of prevalent and incident CVD (OR: 1.81, CI: 1.23 - 2.66) and (HR: 1.37, CI: 1.10 - 1.72) respectively, but no association was reported between it and the cardiovascular overall mortality [23].

Our current study yielded similar relationship between S2-S3 NAFLD and CVD (OR: 24, CI: 17 - 31), and despite the smaller number of included patients in our study, the used tools for diagnosis of NAFLD and CVD; either transient elastography and coronary angiography, are more accurate and objective than those in the previous two studies [21] [22] that were observational and depended only on the history for diagnosis of CVD (coronary, cerebrovascular, and peripheral vascular disease), and abdominal US for diagnosis of NAFLD. **Figure 3** shows an example of 33-year-old male patient with S3 liver steatosis (CAP = 368) and his angiography findings showed significant stenosis at the left circumflex coronary artery (LCX), chronic total occlusion at the left anterior descending (LAD) and normal right coronary artery (RCA), with moderate-severity coronary ischemia as indicated by Gensini score of 56. Amazingly; this patient had a body mass index of 31 kg/m^2 with no other cardiovascular risk factors.

In contrary to the above-mentioned data, NAFLD as estimated by fatty liver index was not a significant predictor of acute myocardial infarction in a long-term prospective study which also emphasized interplay of confounders [24]. Another large-scale prospective study by Chang *et al.* has shown similar findings with insignificant association between NAFLD and CVD hospitalization after further adjusting for potential mediators [25].

The discrepancy between the results of the latter two studies and the previous ones could be assumed to the wide array of cardiovascular presentations that considered in each study as well as the variable methods of NAFLD diagnosis which included the US, computerized tomography and non-invasive markers.

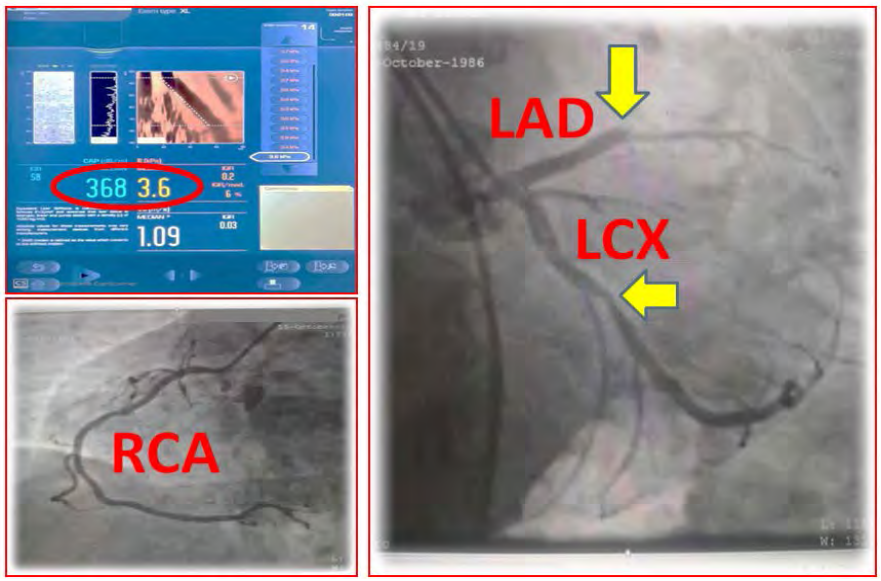

Figure 3. Transient elastography of 33-year-old male patient with S3 liver steatosis (CAP = 368 dB/m), F0-F1 fibrosis (3.6 kilopascal); both appear red-encircled, and angiographic findings showed significant stenosis (yellow horizontal arrow) at the left circumflex coronary artery (LCX), chronic total occlusion (yellow vertical arrow) at the left anterior descending (LAD) and normal right coronary artery (RCA), with calculated Gensini score of 56.

The main advantage of our study is using CAP as a diagnostic tool for NAFLD which allows not just diagnosis of NAFLD but also its stratification from S0 to S3, and coronary angiography with Gensini score calculation which reflects more realistic assessment of the cardiovascular risk. However, we had certain limitations including small sample size and lack of long-term follow up.

5. Conclusion

In conclusion, NAFLD is an independent cardiovascular risk predictor with statistically significant increase in Gensini score with the higher grades of NAFLD.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

CAP: Controlled Attenuation Parameter.
CLD: Chronic Liver Disease.
CVD: Cardiovascular Disease.
LSM: Liver Stiffness Measurement.
NAFLD: Non-Alcoholic Fatty Liver Disease.
NASH: Non-Alcoholic Steatohepatitis.
TE: Transient Elastography.
US: Abdominal Ultrasound.

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Effects of Semi-Solid Enteral Formula on Aspiration Pneumonia and Diarrhea

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Abstract

Objective: The effects of semi-solid enteral formula were investigated in tube feeding patients with aspiration pneumonia and/or diarrhea caused by liquid enteral formula. **Methods:** In 25 cases of aspiration pneumonia and 10 cases of diarrhea (5 cases had both aspiration pneumonia and diarrhea) caused by liquid enteral formula, the rate of improvement by changing the liquid enteral formula to semi-solid enteral formula was studied. The semi-solid enteral formula (PG Soft® EJ) was infused via the nasogastric tube (16Fr) or percutaneous endoscopic gastrostomy (PEG) tube (20Fr). **Results:** The semi-solid enteral formula was effective in 72% of aspiration pneumonia cases and in 80% of diarrhea cases. Constipation was observed in one case but was controlled with magnesium oxide. **Conclusion:** In cases of aspiration pneumonia and/or diarrhea, changing liquid enteral formula to semi-solid enteral formula frequently shows improvement.

Keywords

Semi-Solid Enteral Formula, Tube Feeding, Aspiration Pneumonia, Diarrhea, Liquid Formula Syndrome

1. Introduction

Gastroenteric tube feeding plays a major role in the management of patients with poor voluntary intake, chronic neurological or mechanical dysphagia or gut dysfunction, and patients who are critically ill [1]. But tube feeding is a risk indicator of aspiration pneumonia [2] and diarrhea is a common and problematic complication of enteral nutrition [3].

In order to reduce the incidence of aspiration pneumonia and/or diarrhea in

tube feeding, modification of the volume and administration method of tube feeding [4], measurement of gastric residual volumes at monitoring intervals of 4 hours [5], placement of feeding tubes postpylorically [6] and use of feeding pump with a continuous infusion for 20 hours adjusting infusion rate based on gastric residual volume [7] have been considered.

Semi-solid enteral formula has been demonstrated to reduce the incidence of aspiration pneumonia but is difficult to administer via the nasogastric tube [8]. We investigated the effects of changing liquid enteral formula to semi-solid enteral formula in patients with aspiration pneumonia and/or diarrhea in whom liquid enteral formula had been infused via the nasogastric tube or PEG tube.

2. Materials and Methods

Patients with liquid enteral formula who showed aspiration pneumonia and/or diarrhea in our hospital were asked to participate in our study. Thirty-one patients agreed to participate but one patient withdrew from our study because symptoms of reflux esophagitis were aggravated not by semi-solid enteral formula but by 16Fr nasogastric tube. All patients were on total enteral nutrition.

The mean age of the 30 patients was 79.5 years (youngest 49, oldest 97 years), female:male ratio was 21:9 and the mean body weight was 47.4 kg. Twenty-five patients showed aspiration pneumonia and 10 patients showed diarrhea (five patients showed both aspiration pneumonia and diarrhea). The enteral formula was infused via the nasogastric tube in 26 patients and via the PEG tube in four patients. The underlining diseases were cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, lung cancer, liver cancer, malignant lymphoma, etc.

This study was approved by the ethics committee of our hospital and we got the informed consent from the patients and/or their family. We changed the liquid enteral formula to semi-solid enteral formula (PG Soft® EJ) and infused via the nasogastric tube (16Fr) or PEG tube (20Fr). We infused 300 - 400 kcal of PG Soft® EJ for 30 minutes using a pressurized bag (**Figure 1**) three times a day. One hundred mL of water was injected 30 minutes before the infusion of PG Soft® EJ and 50 mL of water was injected after the infusion of PG Soft® EJ via the nasogastric tube or PEG tube. The liquid enteral formula was Isocal® Bag 2K in 25 cases, Peptamen® in 3 cases, Isocal® Support Bag in 1 case and Renalen® D in 1 case.

Diarrhea was defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual [9]. The effects of PG Soft® EJ were classified into improvement, worsening, and no effects.

3. Results

Table 1 shows the effects of changing liquid enteral formula to PG Soft® EJ. Eighteen out of the 25 cases of aspiration pneumonia and 8 out of the 10 cases of diarrhea showed improvement. None of the 30 cases showed worsening. A case

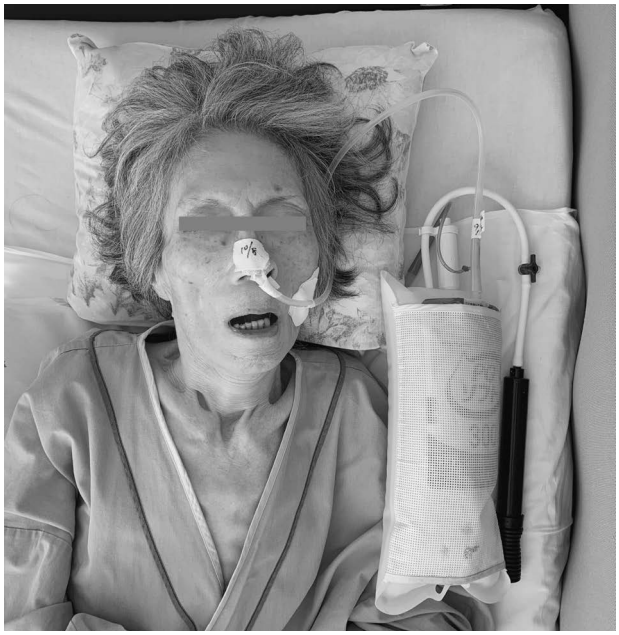

Figure 1. Method of administration of PG Soft® EJ. The semi-solid enteral formula was infused with a pressurized bag which may be placed on the bed.

Table 1. The effects of changing liquid enteral formula to PG Soft® EJ.

	Improvement	Worsening	No Effects
Aspiration Pneumonia	72%	0%	28%
Diarrhea	80%	0%	20%

of aspiration pneumonia developed constipation which was controlled with magnesium oxide. Besides that, no adverse effects were noted.

4. Discussion

Liquid formula syndrome is various complications of liquid enteral formula which are due to low viscosity [10]. Some clinical complications that can occur with enteral nutrition, such as diarrhea and gastroesophageal reflux, are observed after administration of a liquid enteral formula and thickened enteral formula has been reported to be able to prevent these complications [11]. Thickened enteral formula is also referred to as semi-solid enteral formula [12]. Aspiration pneumonia is presumably due to severe gastroesophageal reflux and semi-solid enteral formula has been demonstrated to reduce the incidence of aspiration pneumonia [8].

The positive effects of thickened enteral formula are considered to be based on its high viscosity, which reduces the outflow rate of gastric contents and thereby helps to prevent diarrhea and gastroesophageal reflux [12]. Thickened enteral formula is a formula in which viscosity is intentionally increased to prevent enteral nutrition-related complications, such as aspiration pneumonia and diarrhea

[12] and has been used mainly in Japan [11]. It has been more than a decade since semi-solid enteral formula was developed but there is limited published literature on this topic despite the wide usage of semi-solid enteral formula in Japan [13]. Semi-solid enteral formula is considered to be more physiologic because swallowed food does not enter the stomach in the liquid form [13].

Semi-solid enteral nutrients have high viscosity and, therefore, are typically administered through a large-diameter tube [11] [14]. We used 12Fr nasogastric tube and 20Fr PEG tube for liquid enteral formula and 16Fr nasogastric tube and 20Fr PEG tube for PG Soft® EJ. One patient withdrew from the study because symptoms of reflux esophagitis were aggravated not by semi-solid enteral formula but by 16Fr nasogastric tube. We could infuse PG Soft® EJ for 30 minutes with the use of a pressurized bag.

Table 2 shows the difference between Isocal® Bag 2K (400 kcal) and PG Soft® EJ (400 kcal). The viscosity is the biggest difference between the two [13]. Several studies examining thickened enteral formula with viscosity ranging from 900 to 20,000 mPa·s have shown the efficacy of thickened enteral formula in preventing gastroesophageal reflux in this range [12]. Several clinical case studies have been published on prevention of diarrhea using thickened enteral formula with viscosity ranging from 3000 to 20,000 mPa·s [12]. The viscosity of Isocal® Bag 2K is 20,000 mPa·s and this viscosity is considered to be effective for prevention of both aspiration pneumonia and diarrhea. We used PG Soft® EJ because of its high viscosity but the other semi-solid enteral formulas with viscosity ranging from 3000 to 20,000 mPa·s [12] are considered to be also effective.

In the case of liquid enteral formula we administered 150 mL of water simultaneously with the liquid enteral formula and 50 mL of water was injected after the infusion of liquid enteral formula to flush the nasogastric tube or PEG tube. But in the case of semi-solid enteral formula, simultaneous administration of water decreases its viscosity. So, we injected 100 mL of water 30 minutes before and 50 mL of water after the infusion of PG Soft® EJ via the nasogastric tube or PEG tube.

Table 2. Comparison between Isocal® Bag 2K and PG Soft® EJ.

	Isocal® Bag 2K	PG Soft® EJ
Energy (kcal)	400	400
Volume or Weight	200 mL	267 g
Water	140 mL	175 g
Protein (g)	14.4	16.0
Fat (g)	16.0	8.8
Saccharides (g)	47.6	62.7
Dietary Fiber (g)	4.0	1.5
Viscosity (mPa·s)	40	20,000

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We got an approval to use the photograph from the patient and her family.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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