Original Article Clinical significance of the distribution of infection and pathogens along with the changes of hemorheology indexes, cortisol, apolipoprotein E and procalcitonin after hip arthroplasty

Erjian Wang, Jinzhu Zhang, Xianguo Luo, Zhi Dou

Department of Orthopedics, Qiqihar First Hospital, Qiqihar, Heilongjiang Province, China Received April 11, 2020; Accepted May 11, 2020; Epub August 15, 2020; Published August 30, 2020

Abstract: Objective: To investigate the clinical significance of the distribution of infection and pathogens, and the changes of hemorheology indexes, cortisol (Cor) and apolipoprotein E (APoE) after hip arthroplasty (HA). Methods: Forty-three patients with infection after HA were selected as the infection group, while 43 uninfected patients after HA during the same period were selected as the non-infection group. Pathogen identification and drug susceptibility test were conducted on the infection group. General information of the two groups was compared, and multivariate Logistic regression analysis was performed for the indicators with statistical differences in univariate analysis. The levels of hemorheology indexes (whole blood high-shear viscosity (WBHSV), whole blood low-shear viscosity (WBLSV), plasma viscosity (PV), erythrocyte aggregation index (EAI), erythrocyte rigidity index (ERI), erythrocyte deformation index (EDI)), APoE, Cor and procalcitonin (PCT) were analyzed in the two groups. A ROC curve was used to analyze the clinical value of each index in the diagnosis of postoperative infection. Results: A total of 52 pathogens were isolated in the infection group, of which 28 were gram-positive bacteria, accounting for 53.85%. Staphylococcus aureus and staphylococcus epidermidis in the gram-positive bacteria had higher drug resistance to penicillin and chloramphenicol; while escherichia coli and enterobacter cloacae both gram-negative bacteria had higher drug resistance to cefazolin and ampicillin. The results of univariate analysis showed that there were statistically significant differences between the two groups in age, operation time, intraoperative blood transfusion volume, serum albumin level, length of hospital stay, antimicrobial use, hormone drug use, history of diabetes, BMI, anemia, and drainage tube placement time (all P<0.05). The results of multivariate Logistic regression analysis showed that age \geq 60 years, length of hospital stay \geq 10 days, combination with diabetes mellitus, operation time \geq 3 hours, no antimicrobial use, hormone drug use, and drainage tube placement time ≥2 days were independent risk factors for postoperative infection. Compared with the non-infection group, the levels of WBHSV, WBLSV, PV, EAI, Cor, APoE and PCT in the infection group were increased significantly, while the levels of EDI and ERI were decreased significantly (all P<0.05). The results of ROC curve showed that each index had certain clinical value in the diagnosis of postoperative infection (all AUC>0.7), and Cor and APoE had higher diagnostic value (both AUC>0.9). Conclusion: Gram-positive bacteria are a major pathogen in postoperative infection after HA. The detections of hemorheology indexes, APoE and Cor have certain diagnostic value for postoperative infection after HA.

Keywords: Hip arthroplasty, postoperative infection, hemorheology, apolipoprotein E, cortisol

Introduction

With the development of modern society and the aggravation of population aging, the incidence of hip-joint disease is increasing annually. However, the decline of various bodily functions in the elderly, such as osteoporosis and slow limb response, leads to an increasing risk of falling or slipping, which further increases the incidence of femoral neck fractures [1-3]. Hip arthroplasty (HA) is a surgical method of implantation of exogenous biomaterials, which has been widely used in clinical practice because of its good therapeutic effects on femoral neck fracture, femoral head necrosis, congenital hip dysplasia and other diseases [4, 5]. However, postoperative infection and other complications of HA can seriously affect the

prognosis of patients. There are many causes of infection after HA, but they are still controversial. Therefore, it is of great importance to explore the distribution of pathogens and its influencing factors [6]. At present, microbial culture is mainly used to diagnose infection after HA in clinic, but it is difficult for clinicians to take effective measures to control the infection due to some of the shortcomings such as long culture periods and many interfering factors. Therefore, it is urgent to explore the specific and sensitive indicators of early infection detection after HA for the diagnosis and treatment of postoperative infection [7]. The hemorheology indexes are mainly used to evaluate the blood rheology and blood viscosity. Blood viscosity indexes include whole blood high-shear viscosity (WBHSV), whole blood low-shear viscosity (WBLSV), plasma viscosity (PV); the increase of their levels indicates that body's blood is in a state of high coagulation and viscosity. Erythrocyte aggregation index (EAI), erythrocyte rigidity index (ERI), and erythrocyte deformation index (EDI) are indexes to evaluate red blood cells, and their level changes are also closely related to blood viscosity. Study has shown that when postoperative infection occurs, inflammatory and stress reactions will cause certain adverse effects on the body. Abnormal manifestations are presented on the microcirculatory system under the influence of stress reactions, and the hemodynamics is changed, resulting in the occurrence of insufficient local blood supply of the body [8]. Therefore, the analysis of hemorheology indexes of postoperative infection has certain clinical significance for the diagnosis of infection. Cortisol (Cor), as one of the indicators of stress response, is involved in the body's inflammatory response process. Research has found that Cor level was significantly increased after HA, so the level change in postoperative infection is also one of the focuses of this study [9]. It was initially found that apolipoprotein E (APoE) played an important role in lipid metabolism; with the deepening of research, it is also found that APoE plays an essential part in the occurrence and development of infectious diseases, and can be regard as an indicator to evaluate the severity of infection and prognosis [10]. Therefore, this study explored the distribution and influencing factors of pathogens, and changes in hemorheology indexes, APoE and Cor of postoperative infection after HA, aiming to provide relevant evidence for clinical effective prevention and control of postoperative infection.

Materials and methods

General information

Forty-three patients with infection after HA admitted to Qiqihar First Hospital from January 2017 to January 2019 were selected as the infection group, while 43 uninfected patients after HA during the same period were selected as the non-infection group. The diagnostic criteria for infection referred to the relevant criteria proposed by the Musculoskeletal Infection Society in 2011 [11]. Patients and their families were informed and signed an informed consent, and this study was approved by the Ethics Committee of Qiqihar First Hospital.

Diagnostic criteria: 1) There were sinus passages going into the prosthesis; 2) The same pathogen was cultured from two soft tissues or extracted effusions around the prosthesis; and 3) The conditions conformed to at least 4 of the following items in accord with the diagnostic criteria, including increased erythrocyte sedimentation rate or C-reactive protein, increased synovial leukocyte count, increased percentage of synovial neutrophils, fester in the affected joints, primary pathogen culture from periarticular tissue or extracted joint fluid, and the existence of more than 5 neutrophils at 5 high power lens (400×) in the pathological analysis of the periprosthetic tissues.

Inclusion criteria: 1) Patients with successful first-time HA; 2) Patients with no previous history of infection; 3) Patients with bone maturation; and 4) Aged from 18 to 80 years.

Exclusion criteria: 1) Patients with other visceral diseases; 2) Patients with incomplete clinical information; patients with active infection in other sites; 4) Patients who died in the perioperative period; 5) Patient with terminal diseases such as malignant tumors; and 6) Patients with poor compliance.

Identification and drug resistance analysis of pathogens

The infected sites of the infection group were sampled, inoculated into the sterile culture bot-

Pathogens	Strains	Proportion (%)				
Gram-positive bacteria	28	53.85				
Staphylococcus aureus	15	28.85				
Staphylococcus epidermidis	7	13.46				
Coagulase-negative staphylococcus	4	7.69				
Enterococcus faecalis	1	1.92				
Streptococcus hemolyticus	1	1.92				
Gram-negative bacteria	24	46.15				
Escherichia coli	9	17.31				
Enterobacter cloacae	7	13.46				
Klebsiella pneumoniae	4	7.69				
Pseudomonas aeruginosa	3	5.77				
Enterobacter aerogenes	1	1.92				

 Table 1. Distribution of pathogens

tles, and then were immediately sent for examination. Streaking inoculation of the samples was performed on the blood plate medium. Some special samples were inoculated in broth medium and then performed streaking inoculation in the blood plate medium. The samples were incubated at 37°C for 24-72 h. Then the bacteria were preliminarily identified according to gram staining results and colony characteristics. Finally, the species identification of bacteria was conducted by a fully automatic microbial identification instrument (MiCoroScan Walk-Away plus 96; Beckman Kurt Co., Ltd., USA). The quality control bacteria were staphylococcus aureus (ATCC25923), staphylococcus epidermidis (ATCC12228) and klebsiella pneumoniae (ATCC70060), all of which were purchased from the laboratory of Chinese Center for Disease Control and Prevention. The drug susceptibility test of gram-positive bacteria was conducted by GN2011 method, and that of gram-negative bacteria was conducted by GP method. The paper-diffusion method was used to test the drug susceptibility.

Outcome measures

General information was compared between the two groups, including age, gender, operation time, intraoperative blood transfusion volume, serum albumin level, length of hospital stay, antimicrobial use, hormone drug use, history of diabetes, BMI, anemia, drainage tube placement time and prosthetic type.

Two tubes of venous blood (5 mL) were collected in the morning of the next day after enrollment, stored in anticoagulant tubes and sent for immediate examination. One tube of venous blood was analyzed with the EB-2600A automatic hemorheology detector (Chongging Yunfan Medical Equipment Co., Ltd., China) to detect hemorheology indexes (WBHSV, WBLSV, PV, EAI, EDI, ERI). The other tube was centrifuged at 3,000 r/min for 5 min to separate the serum. The levels of procalcitonin (PCT), APoE and Cor were detected by enzyme-linked fluoroimmunoassay (Spectra-MaxParadigm multi-mode microplate reader: Molecular Devices Corporation, USA), immunoturbidimetry (MINDRAY BS-800 automatic biochemical analyzer; Shenzhen Mindray Biomedical Electronics Co., Ltd., China) and

chemiluminescence immunoassay (Beckman Coulter UniCel DxI800 fully automatic chemiluminescence analyzer; Beckman Coulter Inc., USA), respectively.

Statistical analysis

Statistical analysis was conducted by SPSS 22.0 software. Count data were expressed as percent (%) and were analyzed by chi-square test. Measurement data were expressed as mean \pm standard deviation ($\overline{X} \pm$ SD) and analyzed by t test. Multivariate Logistic regression analysis was performed for the indicators with statistical differences in univariate analysis. ROC curve was used to analyze the clinical value of hemorheology indexes, APoE, Cor and PCT in the diagnosis of postoperative infection. An AUC value between 0.5 and 0.7 indicated low accuracy, between 0.7 and 0.9 certain accuracy, and between 0.9 and 1.0 high accuracy. P<0.05 was considered statistically significant.

Results

Distribution of pathogens

A total of 52 pathogens were isolated in the infection group, of which 28 were gram-positive bacteria (53.85%) and 24 were gram-negative bacteria (46.15%). See **Table 1**.

Comparison of antibacterial resistance between main gram-positive and gram-negative bacteria

Staphylococcus aureus and staphylococcus epidermidis in the gram-positive bacteria had

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	Staph	ylococcus aureus (n=15)	Staphylococcus epidermidis (n=7)		
Antibacterial agents	Strains Drug resistance rate (%)		Strains	Drug resistance rate (%)	
Penicillin	14	93.33	7	100.00	
Chloramphenicol	12	80.00	5	71.43	
Erythromycin	7	46.67	3	42.86	
Ciprofloxacin	6	40.00	3	42.86	
Oxacillin	4	26.67	2	28.57	
Levofloxacin	3	20.00	2	28.57	
Vancomycin	0	0	0	0	

Table 2. Drug resistance results of major gram-positive bacteria

Table 3. Drug resistance results of major gram-negative bacteria

Antibacterial agents	E	scherichia coli (n=9)	Enterobacter cloacae (n=7)		
	Strains	Drug resistance rate (%)	Strains	Drug resistance rate (%)	
Cefazolin	9 100.00		7	100.00	
Ampicillin	7	77.78	6	85.71	
Amoxicillin	5	55.56	5	71.42	
Ceftazidime	3	33.33	2	28.57	
Levofloxacin	2	22.22	2	28.57	
Piperacillin	1	11.11	1	14.29	
Amikacin	0	0	1	14.29	
Imipenem	0	0	0	0	

higher drug resistance to penicillin and chloramphenicol; while escherichia coli and enterobacter cloacae in the gram-negative bacteria had higher drug resistance to cefazolin and ampicillin. See **Tables 2**, **3**.

Comparison of general information

The results of univariate analysis showed that there were statistically significant differences between the two groups in age, operation time, intraoperative blood transfusion volume, serum albumin level, length of hospital stay, antimicrobial use, hormone drug use, history of diabetes, BMI, anemia, and drainage tube placement time (all P<0.05). See **Table 4**.

Results of multivariate logistic regression analysis

It showed that age \geq 60 years, length of hospital stay \geq 10 days, combination with diabetes mellitus, operation time \geq 3 hours, no antimicrobial use, hormone drug use, and drainage tube placement time \geq 2 days were independent risk factors for postoperative infection. See **Table 5**.

Comparison of hemorheology indexes, Cor, APoE and PCT levels

Compared with the non-infection group, the levels of WBHSV, WBLSV, PV, EAI, Cor, APoE and PCT in the infection group were increased significantly, while the levels of EDI and ERI were decreased significantly (all P<0.05). See **Table 6.**

ROC curve results

The results of ROC curve showed that each index had certain clinical value in the diagnosis of postoperative infection (all AUC>0.7), and Cor and APoE had higher diagnostic value (AUC=0.930, AUC=0.909). See **Table 7** and **Figure 1**.

Discussion

HA is an operative method to relieve patients' pain and improve their quality of life by replacing diseased joints with metal, ceramic and other novel materials. Although this approach has made great progress, its complications, such as infection and pain, still brings serious impacts to patients [12, 13]. The results of this

Factors	Infection group (n=43)	Non-infection group (n=43)	X ²	Р
Age			4.778	0.029
≥60 years	30	20		
<60 years	13	23		
Gender			0.048	0.826
Male	25	26		
Female	18	17		
Operation time			4.674	0.031
≥3 h	28	18		
<3 h	15	25		
Intraoperative blood transfusion volume			7.167	0.007
≥800 mL	22	10		
<800 mL	21	33		
Serum albumin level			6.790	0.009
≥35 g/L	18	30		
<35 g/L	25	13		
Length of hospital stay			10.673	0.001
≥10 d	26	11		
<10 d	17	32		
Antimicrobial use			17.751	<0.001
Yes	7	26		
No	36	17		
Hormone drug use			11.913	<0.001
Yes	30	14		
No	13	29		
History of diabetes			4.914	0.027
Yes	12	4		
No	31	39		
BMI			3.903	0.048
≥25 kg/m²	30	21		
<25 kg/m ²	13	22		
Anemia			4.977	0.026
Yes	21	11		
No	22	32		
Drainage tube placement time			7.005	0.008
≥2 d	23	11		
<2 d	20	32		
Prosthetic type			0.443	0.506
Bone cement type	15	18		
Bone cementless type	28	25		

 Table 4. Comparison of general data between the two groups (case)

study showed that gram-positive bacteria were the main pathogens of infection, accounting for 53.85%, which was consistent with previous research results [13]. The possible reason was that gram-positive bacteria formed biofilms on the surface of the prosthesis, which were concentrated in the interosseous tissues and bone-prosthesis junction; therefore, leading to the antibacterial failure owing to the impermeation of antibiotics. The results of drug resistance analysis showed that the strains infected after HA had higher drug resistance in general. Staphylococcus aureus and staphylococcus epidermidis in the gram-positive bacteria had

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Factors	β	SE	Wald χ^2	Р	OR	95% CI
Age ≥60 years	0.317	1.164	4.325	0.021	1.443	1.017-1.893
Combination with diabetes mellitus	2.663	4.798	9.986	<0.001	1.874	1.021-3.325
Length of hospital stay ≥10 d	2.137	3.344	5.786	0.008	1.243	1.013-4.492
Operation time \geq 3 h	0.289	1.819	5.734	0.013	1.338	1.083-1.498
No antimicrobial use	0.957	0.532	6.034	0.011	2.604	1.512-2.987
Hormone drug use	1.876	3.686	11.345	<0.001	2.165	1.398-4.018
Drainage tube placement time $\ge 2 \text{ d}$	0.421	1.718	4.984	0.029	1.518	1.108-2.046

Table 5. Results of multivariate Logistic regression analysis

Indicators	Infection group (n=43)	Non-infection group (n=43)	t	Р
PV (mPa/s)	1.85±0.29	1.45±0.25	6.889	<0.001
WBLSV (mPa/s)	7.33±1.65	5.19±0.79	7.676	<0.001
WBHSV (mPa/s)	8.26±1.75	6.29±0.79	6.699	<0.001
EAI	4.69±0.82	3.39±0.76	7.658	<0.001
ERI	3.73±0.51	4.62±0.76	6.396	<0.001
EDI	0.42±0.08	0.56±0.11	6.527	<0.001
Cor (nmol/L)	431.72±88.62	277.30±58.68	9.527	<0.001
APoE (mg/L)	55.14±10.89	35.03±8.53	9.530	<0.001
PCT (µg/L)	3.57±0.94	2.23±0.72	7.418	<0.001

Note: PV, plasma viscosity; WBLSV, whole blood low-shear viscosity; WBHSV, whole blood high-shear viscosity; EAI, erythrocyte aggregation index; ERI, erythrocyte rigidity index; EDI, erythrocyte deformation index; Cor, cortisol; APoE, apolipoprotein E; PCT, procalcitonin.

Indicators	Cut-off value	AUC	95% CI	Sensitivity	Specificity	Р
PV (mPa/s)	1.777	0.838	0.756-0.921	0.674	0.837	< 0.001
WBLSV (mPa/s)	6.333	0.853	0.764-0.941	0.791	0.930	<0.001
WBHSV (mPa/s)	7.487	0.814	0.714-0.914	0.744	0.930	<0.001
EAI	4.183	0.872	0.789-0.955	0.814	0.907	<0.001
ERI	4.727	0.819	0.730-0.908	0.977	0.558	<0.001
EDI	0.524	0.829	0.742-0.917	0.930	0.651	<0.001
Cor (nmol/L)	323.500	0.930	0.878-0.981	0.907	0.837	<0.001
APoE (mg/L)	49.675	0.909	0.846-0.972	0.721	0.977	<0.001
PCT (µg/L)	2.583	0.873	0.800-0.946	0.837	0.791	<0.001

Note: PV, plasma viscosity; WBLSV, whole blood low-shear viscosity; WBHSV, whole blood high-shear viscosity; EAI, erythrocyte aggregation index; ERI, erythrocyte rigidity index; EDI, erythrocyte deformation index; Cor, cortisol; APoE, apolipoprotein E; PCT, procalcitonin.

higher drug resistance to penicillin and chloramphenicol; while escherichia coli and enterobacter cloacae in the gram-negative bacteria had higher drug resistance to cefazolin and ampicillin. It indicates that there are some differences in the drug resistance of pathogens in the secondary infection caused by different pathogens, which has certain clinical value in the guidance of rational drug use. The influencing factors of infection after HA are extraordinarily complex, including the patient's internal and external factors [14]. Recently, with the increasing awareness of risk factors for infection, the incidence of postoperative infection has presented a decreasing trend. However, due to the influence of patients' own conditions, there is still a common occurrence of postoperative infection. The results of this

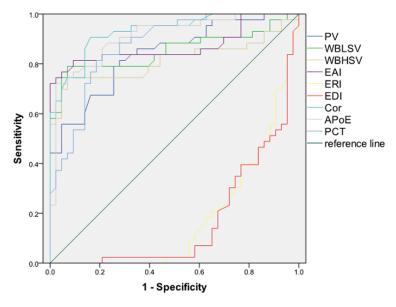


Figure 1. ROC curve results. PV, plasma viscosity; WBLSV, whole blood lowshear viscosity; WBHSV, whole blood high-shear viscosity; EAI, erythrocyte aggregation index; ERI, erythrocyte rigidity index; EDI, erythrocyte deformation index; Cor, cortisol; APoE, apolipoprotein E; PCT, procalcitonin.

study showed that age ≥ 60 years, length of hospital stay ≥10 days, combination with diabetes mellitus, operation time ≥ 3 hours, no antimicrobial use, hormone drug use, and drainage tube placement time ≥ 2 days were independent risk factors for postoperative infection. The decline of various body functions causes the weakening of immunity in the elderly patients, leading to the decline of ability to resist bacterial invasion. In addition, the elderly patients are afraid of surgical treatment, and great psychological pressure will further lead to the decline of patients' bacterial resistance. Patients with diabetes have poorer bone condition than the normal, which probably results from an increased risk of osteoporosis caused by inadequate insulin production. Besides, high blood glucose can also lead to osmotic diuresis. Also micturition can promote the excretion of calcium and phosphorus, leading to bone loss, thereby increasing the risk of femoral neck fracture. However, the environment with high level of blood glucose is conducive to the propagation of pathogens, thereby increasing the risk of postoperative infection [15, 16]. Prolonged operation time, length of hospital stay or drainage tube placement time can lead to prolonged exposure, increasing the risk of pathogenic microbial infection. Patients who take hormone drugs for a long period of time often have skin or connective tissue problems, and continued hormone drug use will increase the risk of fungal or bacterial infections. It showed that no antimicrobial use is one of the risk factors for postoperative infection, which also suggests the necessity of rational selection of antibiotics to prevent infection.

During HA, long-term dislocation, traction and artificial prosthesis compression are required for patients, causing lower limb or local tissue ischemia, and abnormal hemorheology indexes, such as significantly increased PV and EDI level, and decreased ERI level. Study has also shown that the abnormal hemorheology indexes may be related to postoper-

ative ischemia/reperfusion injury, as well as the increased types and levels of circulating immune complexes, promoting the release of oxygen free radicals and leading to erythrocyte damage [17]. The hemorheology indexes will gradually improve to the normal level when the erythrocyte damage is recovered and the inflammatory response is controlled. Increased EAI levels can cause increased capillary resistance and affect blood flow velocity to make the plasma highly viscous, and thus affect blood perfusion. Previous studies have mostly focused on the correlation between hemorheology indexes and deep vein thrombosis after HA [18], while there are few reports on postoperative infection. In this study, compared with the non-infection group, the levels of WBHSV, WBLSV, PV and EAI in the infection group were increased significantly, while the levels of EDI and ERI were decreased significantly. The results of ROC curve showed that hemorheologv indexes had certain clinical value in the diagnosis of postoperative infection (all AUC>0.7). This also suggests that hemorheology indexes of infected patients after HA is in an abnormal state; hemorheology indexes have the advantages of convenient sampling and simple detection, which can be used as one of the reference indicators for the diagnosis of postoperative infection.

PCT is the peptide precursor of calcitonin and normally has extremely low levels [19]. However, once the patient is infected, stimulated by endotoxin and various inflammatory factors, the body secretes a large amount of PCT. leading to a significant increase in the serum PCT level. The abnormality can be detected at 2 h after infection, and the peak value can be reached at 12-24 h after infection. The change of PCT level is also closely related to the severity of infection [20]. Data showed that the increase of PCT level was mainly caused by bacterial, fungal and parasitic infections, while it was not caused by viral infections or other factors; therefore, this indicator was considered to be of high clinical value in the diagnosis of pulmonary bacterial infections [21]. Cor is one of the stress response factors mediated by the hypothalamic-pituitary-adrenalin axis of the body, and its level changes are closely related to anesthesia, surgery and postoperative infection. Cor is involved in the body's immune and inflammatory responses. It was used as one of the indicators for the diagnosis and treatment effect evaluation of elderly critically ill patients with ideal effects [22, 23]. Thus, Cor was adopted as one of the indicators to explore its significance in postoperative infection after HA in this study. ApoE plays an important role in lipid transport and metabolism, and it regulates the body's blood lipid levels. Its elevated levels can lead to damage in the body's vascular walls, increasing the risk of postoperative venous thrombosis. Recent research has found that ApoE is involved in immune regulation of infectious diseases, mainly by inhibiting the expression of metallothionein in the tissues, regulating amyloid-beta-induced inflammatory response, activating redox-sensitive transcription factors, namely nuclear factor kB (NF-kB), ultimately promoting inflammatory response. It suggests that ApoE level can be an indicator of infection [24]. The results of this study showed that compared with the non-infection group, the levels of Cor, APoE and PCT in the infection group were increased significantly, and Cor and APoE had higher diagnostic value in the diagnosis of postoperative infection (AUC=0.930, AUC=0.909). This also indicates that Cor and APoE can be used as auxiliary diagnostic indicators.

Considering that this study was based on a small sample size and lacked dynamical obser-

vation for the changes of each index, multi-center studies with large sample size should be conducted for further confirmation so as to provide relevant basis for clinical prevention and diagnosis of infection after HA.

To sum up, gram-positive bacteria are the main cause of postoperative infection after HA, so corresponding treatment schemes should be adopted according to the different pathogens of infection. This study found that the elderly patients ≥60 years old or patients with a history of diabetes tended to have increased risk of postoperative infection, and no antimicrobial use or use of hormone drug is also a risk factor for postoperative infection. It suggests that clinicians should actively take measures to prevent and control infection. In addition, prolonged operation time (\geq 3 h) or drainage tube placement time (≥ 2 d) will also increase the risk of infection. Therefore, the operation time and drainage tube placement time should be controlled to reduce the risk of infection. The detections of hemorheology indexes, APoE and Cor are often characterized by simple operation and low cost, and the research results showed that they had certain diagnostic value in the diagnosis of postoperative infection after HA. This provides a new idea for clinical selection of indicators with high specificity and sensitivity.

Disclosure of conflict of interest

None.

Address correspondence to: Zhi Dou, Department of Orthopedics, Qiqihar First Hospital, No.30 Gongyuan Road, Longsha District, Qiqihar 161000, Heilongjiang Province, China. Tel: +86-0452-2459817; E-mail: douzhi7ub2@163.com

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