Article

Serum Amyloid A was correlated with Osteonecrosis of the femoral head by affecting bone metabolism

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Abstract: SAA is an acute phase protein that elevates under inflammatory circumstance. The serum level of SAA was associated with the progression of inflammation in numerous diseases. However, little attention was paid to the correlation between SAA and ONFH. In this study, SAA was found risen in the femoral head of osteonecrosis patients through proteomics analysis and further confirmed by ELISA. Furthermore, SAA was proven affecting bone metabolism in rBMSCs. It facilitated the proliferation of rBMSCs whereas it suppressed the osteogenic differentiation of rBMSCs and accelerated the adipogenic differentiation of rBMSCs. Thus, we deem that serum amyloid A, which is a vital acute phase protein in inflammation, affected bone metabolism and plays an imperative role in the pathophysiological process of ONFH.

Keywords: SAA, ONFH, proteomics, Wnt/ β -catenin, PPAR γ

1. Introduction

ONFH was widely known as a disabling and progressive skeletal disorder, lacking of effective treatment, which can ultimately lead to total hip replacement[1]. The pathogenesis of ONFH remains unclear. Previous studies indicated that progression of ONFH was correlated with ischemia, reduction of osteogenesis, fat embolism which subsequently cause the femoral head collapse[2]. Previous studies suggested the risk factors of ONFH, including glucocorticoid, alcohol, trauma, smoking, various chronic diseases and genetic predilection, were still complicated[3-7]. In the light of recent research, the pathogenesis of ONFH was linked with the severe degradation of bone tissue[8], the disruption of balance between osteogenesis and adipogenesis of BMSCs[9-11] and the acceleration of osteoblast/osteocyte apoptosis[12]. However, the underlying molecular mechanism of ONFH which has studied for years has not been completely elucidated yet.

SAA, a common terminology for a family of acute-phase proteins which is induced by cytokine and synthesized primarily in liver, is coded for by various genes which have a high degree of allelic variation and mammalian homology[13]. Clinically, SAA is thought as a crucial marker of inflammation and a precursor protein of amyloidosis which serves for cellular cholesterol homeostasis, promotion signalling cascades and modulation of intercellular calcium levels[14-17]. Past studies showed that the increase of SAA level was found in broad spectrum of disease as infection, atherosclerosis, COPD, rheumatic arthritis, tumour et al[18-22]. Since SAA harboured the capability of inducing cell migration and adhesion, it played a crucial role in inflammation.[23] In addition, It was also proved producing by inflamed synovial tissue[24] and inducing the destruction of bone and cartilage[25]. Furthermore, studies on SAA and bone metabolism proposed that SAA inhibits RANKL-induced differentiation of BMMs to osteoclast and maintaining macrophage function [26, 27].

In addition, proteomics, which has been long used to authenticate novel proteins from diseased tissues, sera and biofluids[28-30], was performed to screen out the variation of proteome expression correlated to rheumatoid arthritis, osteoarthritis ,osteoporosis and ONFH[31-34]. However, proteomic research in investigating the mutative expression of sera proteins in ONFH patients was still under-researched.

In this study, the serum proteome technology was carried out to assess the difference of sera proteins' expression between ONFH patients and healthy volunteers. In accordance with the proteomics' result, SAA was hypothesized that harboured correlations with ONFH and the assumption was confirmed using ELISA. Then the influence of SAA on the osteogenic and adipogenic differentiation of BMSCs was projected to confirm the link between SAA and ONFH. This work will help us to find in-depth knowledge of SAA induced bone metabolism disorder and the pathogenesis of ONFH.

2. Results

2.1. Ascending levels of SAA in patients with ONFH

Protein profiles from the sera of 11 steroid-induced ONFH patients and from the sera of 11 healthy volunteers were analyzed by 2-DE and stained with silver. 21 proteins spots were found prominently and accordantly different between the two groups, among which 3 proteins upregulated in steroid-induced ONFH patients and 18 proteins downregulated in steroid-induced ONFH patients (Fig 1). The spots determinated in the steroid-induced ONFH group and health control group were replicable. The spots of interest were then digested and analyzed. On the ground of data shown in Table 1, the no.21 spot which upregulated in steroid-induced ONFH patients was identified as serum amyloid A protein. The mass spectrometric characteristics of the other identified proteins were summarized in supplementary materials (Table A2). Subsequently, the serum level of SAA was measured by ELISA of which the result indicated that the SAA serum level ascended in the steroid, alcohol and trauma induced ONFH patients, compared with the health controls. However, no remarkable distinction was able to calculate among the three ONFH groups.

Figure 1. A pair of representative 2-DE gels from the human sera of 11 healthy controls (a) compared with those of 11 steroid-induced ONFH patients (b). The circles in the images indicated the spots showed significant and consistent difference between two groups and SAA protein was pointed out by the arrows.

Table 1. The proteins that changed significantly in the sera of steroid-induced ONFH patients compared with healthy controls.

Spot no.	PI	MW	Protein score	Accession No.	Protein name
21	6.28	13580.5	292	IPI00552578	SAA1; SAA2 Serum amyloid A protein

Figure 2. SAA serum level of steroid-induced, alcohol-induced, trauma-induced ONFH patients and healthy volunteers.

2.2. SAA facilitates the proliferation of rBMSCs

To examine the effect of SAA on the proliferative rate of rBMSCs, the CCK-8 assay was performed at day1, day3 and day7. By comparison of the absorbance measured at 450nm, the ability of SAA on promoting rBMSCs' proliferation was shown by Figure 3. After 3 days' incubation with variable concentration of SAA, the rBMSCs treated with 1µg/ml or 0.1µg/ml SAA showed more intense proliferation comparing with the control group, rBMSCs treated with normal culture medium. Furthermore, the proliferative rate of rBMSCs exposed to concentration gradient was enhanced obviously at day 7. Additionally, the peak in the proliferative rate at day1, day3 and day7 was observed at the concentrations of 0.1µg/ml. Together, these results indicate that the proliferation of rBMSCs is facilitated by SAA.

Figure 3. Cell viability of rBMSCs co-cultured with 0 µg/ml, 1 µg/ml, 0.1 µg/ml or 0.01 µg/ml SAA. *p<0.05, all experiments were performed in triplicate.)

2.3. SAA inhibits the osteogenic differentiation of rBMSCs in vitro

During the osteogenic induction, rBMSCs were treated with different concentration of SAA. After 14 days' incubation, the alizarin red staining and quantification analysis were performed to evaluate the mineralization of cells. As Fig 4a and 4b shown that rBMSCs conditioning with co-incubation of SAA presented feeblish mineralization than cells cultured without SAA, which demonstrated the depressor effect of SAA on osteogenic differentiation. In agreement with the result of alizarin staining, the BCIP/NBT staining equally revealed that the bluish coloration of rBMSCs cultured with SAA was sparser than that without SAA (Fig 4c). Then the the mechanism behind the inhibitory effect of SAA on osteogenesis was investigated using western blot analysis which turned out that the phosphorylation of GSK3β decreased which downregulated the expression of β-catenin due to the addition of SAA, which further proved the result before (Fig 4d). Real time RT-PCR was performed to monitor the expression of osteogenesis related genes, of which the result indicated that the feebler expression of ALP, Runx2, OCN and COL-1 was observed among the groups treated with SAA than the normal osteogenic induction group (Fig 4e-h). Furthermore, little difference was found among different groups, different concentration of SAA.

Figure 4. Osteogenic markers were attenuated by SAA. a. Alizarin red staining. b. Quantification of Alizarin staining. c. NBT/BCIP staining. d. The stable cell lines were subjected to Western blot and detected for GSK3β, p-GSK3β and β-Catenin, normalized to GAPDH. The expression of (e) ALP, (f) Runx2, (g) COL-1 and (h) OCN at the mRNA level. *p<0.05, all experiments were performed in triplicate.

2.4. SAA promotes the adipogenic differentiation of rBMSCs in vitro

The expression of aP2, PPARγ and Adipoq of rBMSCs which experienced adipogenic induction and treated with various doses of SAA, was measured by Real time RT-PCR. As the Fig 5a-c shown clearly, the expression of the biomarkers mentioned before was upregulated by the stimulation of SAA obviously. Erk, one of the MAPK family, was well established that played an important role in

adipogenesis. In the light of Fig 5d and 5e, the phosphorylation of Erk1/2 reduced with the addition of SAA, which acted as the goad to the increase of the expression of PPAR γ . For visualization of the promoting effect, the oil red staining was performed and as the result turned out in Fig 5f that the red stained lipids droplets of rBMSCs which were co-cultured with different concentration of SAA were more numerous than those of the general adipogenic induced group.

Figure 5. Adipogenic markers were reinforced by SAA. The expression of (a) Adipoq, (b) aP2 and (c) PPAR γ at the mRNA level. d. The stable cell lines were subjected to Western blot and detected for Erk1/2, p-Erk1/2 and PPAR γ , normalized to GAPDH. e. The intensity of relative proteins. f. Oil red O staining. *p<0.05, all experiments were performed in triplicate.

3. Discussion

Since the discovery and identification of ONFH, the underlying molecular mechanism of the disease is still an enigma. Although MRI has made a massive contribution to the early diagnosis of ONFH, the failure to evaluate the activity status and prognosis of the disease results in that numbers of patients miss the opportunities for timely treatment. The proteomics technology has been used to analyze the sera proteins levels which altered in ONFH patients, of which the aim is to explore the biomarkers of ONFH[35]. Previous studies have established the relationship between matrix protein, angiogenic molecule and the occurrence of ONFH in experimental animal models[36, 37]. However, lack of proteomic research in investigating the mutative expression of sera proteins in ONFH patients still exists.

In the present study, the proteomic analysis was performed to evaluate the changed expression level of proteins. To significantly improved the reproducibility, sensitivity and objectivity of the researchs, the sample selection criteria were strictly followed, and high abundance proteins were removed to avoid masking mask the low abundance proteins. According to the result, 21 proteins were found varied in steroid-induced ONFH patients compared with healthy volunteers. Although 18 proteins of those were identified, the stress of this study was laid on serum amyloid A which showed upregulation in the serum of patients with steroid-induced ONFH than that of normal subjects.

SAA is present in the blood of healthy individuals at a low expression level, generally found at 20-50 mcg/ml, but can increase to about 1000-fold in 24h after the onset of acute-phase response (ARP) which includes a series of physiologic changes that occurs as a consequence of infection, inflammation, trauma and other events[38-40]. Initially, the liver was considered as the sole source of SAA, but the SAA proteins' synthesis was found in the other tissues as macrophages, adipocytes, kidney and lung[41]. And the transcription of SAA was also discovered in synovial cells and mammary gland[42-44]. In the pre-clinical and early stage of ONFH, the necrosis of bone marrow and osteocytes, the absorption of necrotic area and the synovial inflammation all can cause focal inflammation[24, 45], which can induce the up-regulation of SAA. Furthermore, the over-active cytokines induced by steroid, alcohol and trauma[46-48] can also achieve the same effect, which explained our datum of ELISA in this study which suggested that SAA proteins upward expressed in all the three groups of ONFH patients.

In previous study, the adipogenesis and osteogenesis of BMSCs was proved to be a significant factor which affected osteonecrosis tremendously. The inhibition of osteogenic differentiation following with the increasing lipid generation decreased the bone formation. Moreover, the blood circulation in femoral head was wrecked by the accumulation of fatty tissue, which eventually result in ONFH[49]. The influence of SAA on bone metabolism was also investigated in this study to explore the role of SAA in the balance between osteogenesis and adipogenesis of BMSCs. In line accordance with our results in this study, SAA proteins harboured the capability of promoting proliferation, curbing osteogenesis and facilitating adipogenesis.

For osteogenic differentiation, Wnt/ β -catenin signal pathway was considered as a mediator[50, 51]. The restraint of β -catenin expression, which is in line with GSK3 β phosphorylation, inhibits ALP activity and mineralization[52]. In this study, the inhibitory effect on osteogenesis of SAA was initially assessed by using BCIP/NBT staining and alizarin red staining as qualitative determination. And then the molecular mechanism of the inhibitory effect was probed and discovered that the addition of SAA proteins down-regulated the phosphorylation of GSK3 β , which repressed the expression of β -catenin from the downstream of the signal pathway. Simultaneously, the expression of ALP, Runx2, OCN and COL-1, which were thought as the biomarkers of osteogenic

differentiation[53-55], was also down-regulated due to the addition of SAA proteins according to the PCR results.

For adipogenic differentiation, the Wnt families are also vital mediators for BMSCs commitment to produce preadipocytes[56]. The conversion of BMSCs to preadipocytes that differentiate into adipocytes is triggered by the enforced expression of PPAR γ which is regulated by both the MAPK/PPAR γ signal pathway and Wnt/ β -catenin signal pathway[57, 58]. Scilicet, the decreased phosphorylation of GSK3 β and Erk1/2 proteins precipitate the up-regulation of PPAR γ protein and facilitate the adipogenic differentiation ultimately, which was demonstrated in this study. Nevertheless, the expression of adipogenesis related genes, not only PPAR γ but also aP2 and Adipoq[53, 59, 60], was detected to further confirmed the promotion effect of SAA on BMSCs' adipogenic differentiation.

In this study, we only confirmed the relevance between SAA and ONFH. Whether SAA was inducing factor or aggravating factors of ONFH remains to be detected. Furthermore, the *vivo* test of SAA effect on bone metabolism was unperformed, which will be implemented in future study. To cooperate the *vivo* experiment, we used rBMSCs but not hBMSCs in this study.

4. Materials and Methods

4.1. Proteomics analysis

4.1.1 Patients

The study was approved by the Institutional Review Board of Shanghai No.6 Hospital in accordance with the principles of Declaration of Helsinki and the informed consent was obtained from each patient (the relevant accession number will be provided during review). For proteome analysis, the steroid-induced ONFH group was consist of 11 patients which received cure of corticosteroid medication, was diagnosed as ONFH with MRI findings and taken no treatment of ONFH. Meanwhile, 11 healthy volunteers matched with gender and age were included as the control group. The clinical features and demographic information of the steroid-induced ONFH group and the control group were summarized in the Table 1. For ELISA, twenty patients with steroid-induced ONFH, twenty with alcohol-induced ONFH, twenty with trauma-induced ONFH and twenty healthy volunteers were enrolled in the study. The clinical features and demographic information of the patients and volunteers were summarized in the appendix A (Table A1).

Table 1. Clinical features and demographic information of healthy volunteers and steroid-induced ONFH patients

Clinical features	Numbers
Healthy volunteers	
Mean age (range, years)	34.00±7.54 (21-45)
Gender (male/female)	5/6
Health status	Good
Total	11
Steroid-induced ONFH patients	
Mean age (range, years)	35.45±8.35 (25-56)
Gender (male/female)	7/4
Location	
Left	1 (9.1%)
Right	2 (18.2%)
Bilateral	8 (72.7%)
Total	11

4.1.2 Preparation of serum samples

5ml of peripheral venous blood was drawn from patients and healthy volunteers on the section of outpatient or in the operation room before general anaesthesia. The blood samples were then

coagulated at 37°C for 15 min and centrifuged at 2000×g for 15 min. Subsequently, the supernatant was collected as serum and stored at -80°C.

4.1.3 Elimination of high abundance proteins and quantification

The serum samples were thawed and attenuated at 37°C. Then the attenuated serum samples were percolated with filter membranes with 0.22µm pore size. The 14 highest abundance proteins were depleted from plasma using the MARS (Agilent Technologies spin columns, US) according to the manufacturer's protocol. Depleted sample was then exchanged into 50 mM ammonium bicarbonate using a VivaSpin concentrator (5000 molecular weight cutoff, Satorius Group, Germany). The proteins of the processed serum samples were quantified by using protein assay reagent kit (Bio-Rad Laboratories Inc, Hercules, CA) and stored at -80°C.

4.1.4 Two-dimensional gel electrophoresis

The serum samples were proceeding to perform on Two-dimensional gel electrophoresis. Briefly, 100µg of each serum samples were loaded onto the IPG strips (GE Amersham, UK), of which pH varied from 3 to 10. The first dimensional isoelectric focusing was implemented as follow: 30v for 12h, 500v for 1h, 1000v for 1h, 8000v for 8h and 500v for 4h, using Ettan IPGphor Isoelectric Focusing System (GE Amersham, UK). The proteins after iso-electrophoresis were subsequently spread by second dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), using Hofer SE 600 (GE Amersham, UK). The second-dimension gel electrophoresis was performed on the 12.5% SDS-polyacrylamide slab gels under the condition of 15mA for 30min and 30mA until the trace of bromophenol blue was 0.5cm from the bottom of the gel.

4.1.5 Silver staining

The silver staining of the gels after electrophoresis was performed according to the modified silver-staining protocol with the Silver Stain PlusOne kit (GE Amersham, UK). The gels were disposed as follow: fixed with 50% methanol and 5% ethylalcohol for 1h, sensitized with sensitizing solution which was consist of 0.2% sodium thiosulphate, 30% methanol and 68g/L sodium acetate for 5 min, washed thrice with distilled water for 5min, incubated with 0.25% silver nitrate for 20 min, rinsed thrice with distilled water for 1min, developed with 0.04% formaldehyde and 2% sodium carbonate by intensively shaking and terminated with 5% acetic acid till the coloration was moderate.

4.1.6 Gel imaging

The silver-stained gels were then scanned by UMax Powerlook 2110XL (GE Amersham, UK) and the spots patterns of the gels' images were matched using Image Master 2D system (Amersham Biosciences). The densities of all the spots matched were standardized with the total protein amount in the gel.

4.1.7 Destaining and trypsin digestion

Then different groups of spots were excised from the gel and rinsed with de-ionized water. The washed gommures were destained with 50µl of solution which was composed of 30mmol/L K₃Fe(CN)₆:100mmol/L Na₂S₂O₃=1:1. After lyophilization, the digestion was performed with 5µl of trypsin (Promega, USA) at 37°C for 20 hours. The enzymatic hydrolysate was extracted subsequently, the residua were added with 100µl of 60% ACN /0.1%TFA, ultrasonic washed for 15 minutes and the cleaned solution was obtained and merged with the enzymatic hydrolysate. The collected fluid was then desalted with Ziptip (Millipore, USA) and frozen dried.

4.1.8 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The dried samples were dissolved in 2µl of 20% acetonitrile and added with the supersaturated CHCA matrix solution of which the solvent was composed of 0.5% TFA and 50% acetonitrile after desiccation of the menstruum. All the samples were then analyzed on 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, USA) of which the scan range was set from 800Da to 4000Da with UV

light at 355 nm from a Nd:YAG laser. The further data were searched against the ipi human protein database with Mascot search engine.

4.1.9 ELISA

The serum level of the identified protein, serum amyloid A, was gauged with ELISA which was performed using the Human SAA ELISA Kit (Anogen, Canada) according to the manufacturer's instructions. The patients and volunteers were enrolled in the study and the sera samples were obtained as mentioned before.

4.2 *Vitro* test

4.2.1 Isolation and culture of BMSCs

rBMSCs were obtained from Sprague-Dawley male rats. Briefly, the marrow from the femur and tibia of the male rats was flushed under aseptic conditions. For isolating rBMSCs, the cell suspension was sifted by a 400-mesh filter. After that the filtered cell suspension was centrifuged for 5 minutes at 4°C. The floating fractions were discarded, and the enriched cells were treated with erythrocyte lysis buffers. Afterwards, rBMSCs were suspended and transferred to culture flasks, in which rBMSCs were cultured with α -MEM (Hyclone, USA), supplemented with 10% FBS (Gibco, USA) and 1/100 penicillin-streptomycin (Gibco). The condition of cells keeping was humidified atmosphere containing 5% CO₂ at 37°C, and the culture medium was replaced every 2 days. After 3-5 passages, rBMSCs were used for subsequent experiments. For osteogenic differentiation, rBMSCs were incubated in a culture medium supplemented with 10⁻² M β -sodium glycerophosphate, 50 μ g/ml L-ascorbic acid and 10⁻⁷ M dexamethasone for 14 days. For adipogenic differentiation, the cells were incubated with the adipogenic induction culture medium (Cyagen, China) for 2 weeks. The experimental protocol was approved by the Research Ethics Committee of the Shanghai Sixth People's Hospital-affiliated Shanghai Jiao Tong University and conducted in compliance with regulations of the Helsinki Declaration.

4.2.2 CCK-8 assay

Direct cell counting was performed to compare the proliferative rate of rBMSCs and the SAA (Peprotech, USA) treated rBMSCs. An amount of 1 $\times$ 10⁴ rBMSCs were implanted on a 96-wells plate and treated with different concentration of SAA (0 μ g/ml, 1 μ g/ml, 0.1 μ g/ml and 0.01 μ g/ml). After a certain time of co-culture, the toxicity and proliferation of cells was detected by CCK-8 kit (Beyotime, China). At each time point (1day, 3days and 7days), the proliferative rate of rBMSCs was assessed according to the manufacturer's instructions.

4.2.3 BCIP/NBT staining

To visualize the ALP activity, the BCIP/NBT staining was used. rBMSCs were seeded into a 24-wells plate at 1 $\times$ 10⁵. rBMSCs were cultured with osteogenic induction culture medium and incubated with various concentration of SAA (0 μ g/ml, 1 μ g/ml, 0.1 μ g/ml and 0.01 μ g/ml) for 7 days. The culture medium was renewed every 2 days. At the time point, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20min at room temperature. Then the fixed cells were re-washed with PBS for three times and treated with BCIP/NBT solution (Beyotime, China) light-resistently for 1h at 37°C. All the images were observed and acquired using a microscope (Leica, Germany) and a digital camera (Canon, Japan).

4.2.4 Alizarin red staining and quantification

Alizarin red staining was widely used to evaluate the osteogenic differentiation of cells according to the calcium deposition in the extracellular matrix which was thought as a marker of early osteogenesis. After co-cultured with osteogenic induction culture medium and SAA for certain days (14days), rBMSCs were fixed with 4% paraformaldehyde for 20 minutes and washed with PBS for three times at 37°C. Then the cells were stained with Alizarin red solution (Cyagen, China) for 30 minutes. The images of general view were captured by a digital camera (Canon, Japan) and the 10x

magnification view was acquired by using a microscope (Leica, Germany). For quantification, the stained Alizarin red was extracted using 10% cetylpyridinium chloride. The extracted solution was added to a 96-wells plate and the absorbance at 584nm was measured on.

4.2.5 Oil Red O staining

Oil red O staining was performed after 14 days' treatment with adipogenic induction culture medium (Cyagen, China), during which different concentration of SAA (0µg/ml, 1µg/ml, 0.1µg/ml and 0.01µg/ml) was added. The cells, after 20 minutes' fixing with 4% paraformaldehyde, were rinsed thrice with PBS and stained with Oil Red O which was consist of PBS and 0.5% oil red O stock solution. The neutral lipids were stained red, which was thought as biomarker of adipogenesis. A microscope (Leica, Germany) was used to take stock of the stained cells and obtain the images of 10x magnification view.

4.2.6 Western Blot Analysis

The rBMSCs cells were plated into a 6-wells plate and cultured until nearly 90% confluence. After that, the cells were induced with suitable culture medium (osteogenesis culture medium or adipogenesis culture medium) and incubated certain concentration of SAA, as mentioned before, for 7 days. Subsequently, the proteins were collected from the combinations of cells lysis buffer, phosphorylation inhibitor and proteinase inhibitor, of which the concentration was quantified using a BCA Protein Assay Kit (Cell Signaling Technology, Shanghai, China). Then equal amounts of proteins (20 micrograms) were separated on SDS-PAGE gels by electrophoresis and transferred to PVDF membranes. The membranes were sealed with blocking buffer for 1h and incubated with the corresponding primary antibody at room temperature. After washing with TBST thrice, the membranes were incubated with HRP-conjugated polyclonal goat antibodies, treated as secondary antibodies, β-actin and GAPDH were treated as internal reference. The erk1/2, phosphor-erk1/2, GSK3β, phosphor-GSK3β and Phospho-Smad1/5 antibodies were purchased from Cell Signaling Technology (Shanghai, China), the BMP-2, β-catenin and β-actin antibodies were purchased from abcam (United Kingdom) and the PPARγ and GAPDH antibodies were purchased from Servicebio (Wuhan, China).

4.2.7 Real time reverse transcription-polymerase chain reaction (Real time RT-PCR)

The mRNA expression of osteogenic differentiation relative gene (ALP, OCN, Runx2 and COL-1) and adipogenic differentiation relative gene (aP2, Adipoq and PPARγ) was assessed by Real time RT-PCR. After 7 days' incubation with appropriate induction culture medium and stimulation with various concentration of SAA, the total RNA was extracted from cells using EZ-press RNA purification Kit (EZ Bioscience, China) and reverse transcribed to generate complementary DNA using 4×Reverse Transcription Master Mix (EZ Bioscience, China). The forward and reverse primers (BioTNT, China) of cDNAs were designed as Table 2. Then 10µl per well of mixture which was consist of 1µl of cDNA, 0.3µl of forward primers, 0.3µl of reverse primers, 5µl of qPCR SuperMix (BioTNT, China) and 3.4µl of distillation-distillation H₂O was loaded onto a 384-wells plate and the Real time RT-PCR was performed on the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, US). The expression of relative gene was normalized to β-actin.

Table 2. List of forward and reverse primers used for RT-PCR

Primer	Forward	Reverse
Alp	5' CGT TGA CTG TGG TTA CTG CTG 3'	5' CTT CTT GTC CGT GTC GCT 3'
OCN	5' CAG ACA AGT CCC ACA CAG CA 3'	5' CCA GCA GAG TGA GCA GAG AGA 3'
COL-1	5' TGT GCG ATG GCG TGC TAT 3'	5' CCT ATG ACT TCT GCG TCT GGT G 3'
Runx2	5' ATC ATT CAG TGA CAC CAC CAG 3'	5' GTA GGG GCT AAA GGC AAA AG 3'

PPAR γ	5' CCT CTC TGT GAT GGA TGA CCA 3'	5' ACA TCC CGT TCA CAA GAG CT 3'
aP2	5' TGA AAC TGA CGA TCA CAC AGG 3'	5' ACA GAA CTC ACT GGG ACC TGG 3'
Adipoq	5' ATG ATA CCA ACT GAC TGC CAC T 3'	5' TTG CTT ACT TTG AGG GTT CTG A 3'
β -actin	5' CCT CTA TGC CAA CAC AGT 3'	5' AGC CAC CAA TCC ACA CAG 3'

4.3 Statistical analysis

SPSS was applied to analyze the data, and the means $\pm$ standard deviation (S.D.) was used to express all the data. Comparisons between different groups were assessed by one-way analysis of variance (ANOVA). P < 0.05 was considered as statistically significant.

5. Conclusions

In this study, SAA was first proved to be one of the proteins whose expression enhanced in steroid-induced ONFH patients comparing with healthy individuals through the proteomic research. We demonstrated that SAA facilitated the proliferation of rBMSCs, promotion of adipogenesis and inhibition of osteogenesis by co-cultured with rBMSCs. The suppression of Wnt/ β -catenin signal pathway and activation of MAPK/PPAR γ signal pathway participated in the effect of SAA on bone metabolism.

In summary, SAA can be considered as a kind of particular protein which affects the bone metabolism and played an important role in the pathological process of ONFH.

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Abbreviations

SAA	Serum amyloid A
ONFH	Osteonecrosis of the femoral head
BMM	Bone marrow-derived macrophage
MRI	Magnetic resonance imaging
ELISA	Enzyme linked immunosorbent assay
MARS	Multiple affinity removal columns system
IPG	Immobilized pH gradient
BMSCs	Bone marrow-derived mesenchymal stem cells
CCK-8	Cell Counting Kit-8
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate /nitro blue tetrazolium
ALP	Alkaline phosphatase
α -MEM	α -Modified Eagle Medium
FBS	Fetal bovine serum
PBS	Phosphate buffer saline
PVDF	Polyvinylidene difluoride
HRP	Horseradish peroxidase

aP2	Fatty acid binding protein
PPAR γ	Peroxisome proliferator-activated receptor γ
Erk	Extracellular regulated protein kinase
MAPK	Mitogen-activated protein kinase

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Table A1. Clinical features and demographic information of steroid-induced ONFH patients, alcohol-induced ONFH patients, trauma-induced ONFH and healthy volunteers.

Clinical features	Numbers
Healthy volunteers	
Mean age (range, years)	34.95±8.86 (21-54)
Gender (male/female)	10/10
Health status	Good
Total	20
Steroid-induced ONFH patients	
Mean age (range, years)	36.20±8.56 (22-55)
Gender (male/female)	13/7
Location	
Left	2 (10%)
Right	1 (5%)
Bilateral	17 (85%)
Total	20
Alcohol-induced ONFH patients	
Mean age (range, years)	39.70±8.45 (26-55)
Gender (male/female)	20/0
Location	
Left	1 (5%)
Right	3 (15%)
Bilateral	16 (80%)
Total	20
Trauma-induced ONFH patients	
Mean age (range, years)	21.35±10.73 (12-54)
Gender (male/female)	15/5
Location	
Left	11 (55%)
Right	9 (45%)
Bilateral	0 (0%)
Total	20

Table A2. The proteins that changed significantly in the sera of steroid-induced ONFH patients compared with healthy controls.

Spot no.	PI	MW	Protein score	Accession No.	Protein name
1	6.67	86847	424	IPI00921523	CFB Isoform 1 of Complement factor B (Fragment)
2	6.41	109332.8	71	IPI00879709	C6 Complement component C6 precursor
4	6.55	52384.6	87	IPI00022488	HPX Hemopexin
5	8.48	31673	582	IPI00431645	HPR 31 kDa protein

6	6.13	38940.5	693	IPI00478493	HPR haptoglobin isoform 2 preproprotein
7	6.13	38940.5	439	IPI00478493	HPR haptoglobin isoform 2 preproprotein
8	6.32	41816.7	164	IPI00479708	IGHM Full-length Cdna clone CS0DD006YL02 of Neuroblastoma of Homo sapiens
9	7.38	38766.4	140	IPI00011264	CFHR1 Complement factor H-related protein 1
10	7.38	38766.4	232	IPI00011264	CFHR1 Complement factor H-related protein 1
11	7.38	38766.4	326	IPI00011264	CFHR1 Complement factor H-related protein 1
13	6.89	194170.1	78	IPI00418163	C4B complement C4-B preproprotein
14	5.64	71474.7	404	IPI00019568	F2 Prothrombin (Fragment)
15	4.86	78174.4	242	IPI00017696	C1S Complement C1s subcomponent
17	6.13	45860.8	237	IPI00641737	HPR Haptoglobin
18	6.13	45860.8	268	IPI00641737	HPR Haptoglobin
19	6.13	45860.8	227	IPI00641737	HPR Haptoglobin
20	6.13	38940.5	467	IPI00478493	HPR haptoglobin isoform 2 preproprotein

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