

Studies on Target-specificity and Biological Activity of *Streptococcus* Serum Antibody and Sulfate Amikacin Conjugates

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ABSTRACT

To investigate the target-specificity and biological activity of *Streptococcus* serum antibody and sulfate amikacin conjugates. The recent used polyethylene glycol 6000 (PEG6000) as the coupling agent to produce Coupled complexes of *Streptococcus* serum antibody and sulfate amikacin. Then, analyzed the antibody being in conjugates specificity which against *Streptococcus*, and the antibody being in conjugates immunogenicity. Besides, we also detected the acute toxicity, antimicrobial activity and bioavailability of sulfate amikacin being in conjugates. As a result, the antibody specific binding to *Streptococcus*, instead of *Escherichia coil*, *Pasteurella* and *Staphylococcus aureus*. Biological activity results showed that coupling decreased *Streptococcus* serum antibody immunogenicity, increased *Streptococcus* serum antibody response sensitivity. Simultaneously, the results indicated that coupling reduced the acute toxicity of sulfate amikacin, improved sulfate amikacin bioavailability and antimicrobial activity of sulfate amikacin. The combination effect on the antibacterial activity of drug and the biological activity of serum antibody is helpful for the practical application of targeted drugs.

Keywords: PEG; *Streptococcus* serum antibody; sulfate amikacin.

1. INTRODUCTION

The concentration of the conventional antimicrobial drugs is low in animal body tissues and body fluids (with a few exceptions, such as brain) [1]. Bacteria are mainly distributed in the target organs when they infect the animals [2]. Even within the target organ, the combination of drugs and bacteria also depends entirely on random collisions. To guarantee the curative effect of drugs, higher drug concentration must be maintained within the bacterial colonies for a prolonged amount of time. Therefore, the antibiotics were given at a high dose within a

certain time period of treatment. As a result, drugs were deposited in tissues, especially in the adipose tissue [3], and formed drug residues. Drug metabolism can cause not only waste but also organ damage [4]. Additionally, some bacteria evolve in the presence of the drugs and form drug resistant strains [5]. Therefore, the development of pathogenic bacteria treatment programs aimed at bacteria-specific molecular targets has become a hot spot of present research.

Because antigens can specifically bind to the antibody, a desired characteristic of antibody

targeting drugs [6,7,8,9,10] is that small drug molecules can couple with specific antibodies and then be delivered to particular pathogenic bacterium multiple times without changing the concentration. This would avoid drug waste caused by normal drug distribution, thereby reducing drug consumption and shortening the course of treatment. We prepared targeted antimicrobial agents through antimicrobial coupling to the antibody molecules, which can significantly improve the drug therapeutic effect and eliminate adverse reactions.

In this study, we prepare *Streptococcus* serum antibody-sulfate amikacin conjugates with polyethylene glycol (PEG6000) as the coupling agent and then evaluate the conjugates' specificity and *Streptococcus* serum antibody and sulfate amikacin biological activity. This study will provide a theoretical and experimental basis for bacteria-targeted drug development. In this study, we conjugated small molecule antibiotics and biomolecule antibodies supramolecularly. We evaluated the bioactivity of the small molecule antibiotics and biomolecule antibodies in the super molecular model. We optimized methods to search for antibiotics and accumulated related data about how to improve the bacterial patterns of antibiotics to provide a solution to resolve the abuse of antibiotics.

2. MATERIALS AND METHODS

2.1 Preparing the Sulfate Amikacin and *Streptococcus* Serum Antibody Conjugates

A *Streptococcus* oil emulsion inactivated vaccine was prepared with *Streptococcus*01026 strain (Purchased from The Institute of Microbiology, Hunan Province, China)and immunized rabbits (Animal experiments were performed following a protocol approved by the Institutional Animal Committee of Hunan Agricultural University) to produce the rabbit *Streptococcus* antisera. The antibody was subsequently purified on a GE Healthcare HiTrap desalting column (G-25) equilibrated in 35 mM sodium citrate with 150 mM NaCl and 2 mM EDTA, pH 6.0. Typically, a 40% to 60% yield of antibody was achieved through this process. Purified antibody was buffer-exchanged into a solution containing 50 mM potassium phosphate and 2 mM EDTA, pH 7.0. sulfate amikacin was dissolved in dimethylacetamide (DMA) and added to the antibody and PEG solution to make a final sulfate amikacin /Antibody/PEG molar ratio of 400:2:9. The reaction was allowed to

proceed for 24 hours at 4°C with mixing. The preparation was usually greater than 95% monomeric as assessed by gel filtration and laser light scattering. The conjugates were checked by electron microscopy with phosphotungstic acid dye staining [11].

2.2 The Effect of the Conjugates on the Biological Activity of *Streptococcus* Serum Antibodies

2.2.1 Comparison of *Streptococcus* serum antibody reactogenicity

2.2.1.1 Conjugate response efficiency assay

The serum antibody and conjugates response efficiency were detected by an indirect ELISA method [12]. *Streptococcus* strain 01026 was embedded by glutaraldehyde and blocked, and the titers of *Streptococcus*, immune rabbit serum antibody and healthy rabbit serum was determined by ELISA. Healthy rabbit serum served as a negative control and physiological saline as the blank control.

2.2.1.2 Conjugate response sensitivity assay

Streptococcus bacteria and colloidal gold labeled serum antibodies [13] were mixed at a 4:1 ratio. The mixture was harvested at different time points and centrifuged at 2000 rpm/min for 30 min. The precipitation was embedded and sliced. The slices were stained with phosphotungstic acid and examined under the EM.

2.2.1.3 Conjugate response specificity assay

E. coli strain C44103, *Streptococcus*01026 strain, *Pasteurella multocida* strain 4401 and *Staphylococcus aureus* strain C26112 were mixed with the conjugates (4:1), respectively. After incubating at room temperature for 30 min, *Streptococcus* serum antibody response specificity was observed by sections after fluorescence staining.

2.3 Comparison of *Streptococcus* Serum Antibody Immunogenicity

2.3.1 Preparation of immune serum

Ten healthy rabbits (1.8 ± 0.2 kg) were randomized into two groups (n = 5 animals/group). Control (*Streptococcus*) and conjugates (1 mg/each) were injected into the rabbits every 15 days. After 21 days, the rabbits were starved and were provided drinking water.

All rabbits were sacrificed by drawing-out all of the blood in their hearts next day. The serum was isolated, incubated at 56°C for 30 min and then passed through a 0.3 µm pore size filter and stored in -20°C.

2.3.2 Detection of *Streptococcus* serum antibody response immunogenicity

The response immunogenicity of the *Streptococcus* serum antibody and conjugates were detected by an indirect ELISA method. *Streptococcus01026* strain was embedded with the carbonate buffer solution and blocked; the titers of immune rabbit serum antibody and healthy rabbit serum were detected by ELISA. Healthy rabbit serum served as a negative control, and physiological saline served as the blank control.

2.4 Conjugates' Effect on Biological Activity of Sulfate Amikacin

2.4.1 Acute toxicity assay

Twenty mice were randomized into two groups (n = 10 animals/group): sulfate amikacin (125 mg/kg body weight) and conjugates (750 mg/kg body weight) were injected intraperitoneally (i.p.) into the mice. Mice were monitored daily for appearance and behavior, dietary wishes, activity behaviors, defecation, central nervous system symptoms and death.

2.5 Antimicrobial Activity Assay *in vitro*

2.5.1 The determination of Minimal Inhibitory Concentrations (MIC)

Sulfate amikacin and conjugates were diluted into a certain concentration by microdilution method (5 mg/mL, 1 mg/mL, 500 µg/mL, 100 µg/mL, 50 µg/mL, 10 µg/mL, 5 µg/mL, 1 µg/mL and 0.5 µg/mL) and added to 96-well plates. 50 µL diluted bacteria liquid ($10^6 \sim 10^7$ /mL) were co-incubated with sulfate amikacin or conjugates at 37°C for 18 h. The lowest drug concentration with no bacterial growth is the minimal inhibitory concentration.

2.5.2 The determination of Minimum Bactericidal Concentration (MBC)

One hundred microliters of the minimal inhibitory concentration were placed into no resistance agar medium and cultured overnight at 37°C. The minimum bactericidal concentration is the highest drug concentration, with less than five bacterial colonies.

2.6 Sulfate Amikacin Activity Assay

2.6.1 Determination of *Streptococcus* LD₅₀

Sixty mice (20 ± 2 g) were divided into 10 groups (n = 6 animals/group). The *Streptococcus* 01026 cultures were diluted with broth medium into 10^{-1} - 10^{10} by a 10 times dilution method and then injected intraperitoneally (i.p.) into the mice (0.2 mL/mouse). The LD₅₀ was calculated by the Karber method [14].

2.6.2 *Streptococcus* treatment animal model

The LD₅₀ dose of *Streptococcus01026* strain was injected into the muscles of 180 mice (20 ± 2 g), and the mice were divided into six groups (n = 30 animals/group). When symptoms appeared, five groups of mice were injected with serum antibodies (0.2 mg), sulfate amikacin (0.2 mg), conjugates (0.4 mg), conjugates (0.2 mg) and conjugate injection (0.1 mg), respectively. Mice were monitored every 12 h for three days.

2.6.3 Sulfate amikacin pharmacokinetic parameters assay

Twenty rabbits (1.8 ± 0.2 kg) were divided into two groups: i.p injection of the control (sulfate amikacin 10 mg/kg body weight) or i.p injection of the conjugates (10 mg/kg body weight). Blood samples were taken from the ear vein of the rabbits at 0, 15, 30, 60, 90, 120, 180, 240, 300 and 360 min after treatment. The samples were centrifuged, and then the plasma concentrations of the supernatants were determined by a microbiological method. Pharmacokinetic parameters were obtained from the plasma concentration-time data treated with the MCP-KP pharmacokinetic program. A two-sample *t*-test was used to compare sulfate amikacin pharmacokinetic parameters in conjugates versus control [14].

3. RESULTS

3.1 Preparing the Conjugates of the Sulfate Amikacin and *Streptococcus* Serum Antibody

To prepare the conjugates of the sulfate amikacin and *Streptococcus* serum antibody, *Streptococcus* serum antibodies, sulfate amikacin and PEG6000 were mixed (400:2:9) and tested by electron microscopy. As shown in Fig. 1, all sulfate amikacin were attached to the antibody molecule. To analyze the stability of the conjugates, the conjugates were stored in 4°C

for 30 d, 90 d and 180 d and observed under EM. Sulfate amikacin was still attached to the *Streptococcus* serum antibody and no free sulfate amikacin (data not shown) was seen.

3.2 Conjugates Specifically Binding *Streptococcus*

To detect the response specificity of the conjugates, *Streptococcus*01026 strain, *E. coli* strain C44103, *Pasteurella multocida* strain 4401, and *Staphylococcus aureus* strain C26112 were respectively mixed with conjugates (1 mg/mL). Fluorescence staining results indicated that the conjugates only bind with *Streptococcus*, not with *Escherichia coli*, *Pasteurella* and *Staphylococcus aureus* (Fig. 3 A-B).

3.3 Coupling Improve *Streptococcus* Serum Antibody Biological Activity

3.3.1 Coupling maintain *Streptococcus* serum antibody reactogenicity

To compare *Streptococcus* serum antibody response efficiency, Serum antibody and conjugate response efficiency were detected by an indirect ELISA. Fig. 2 indicated that the ELISA value of the conjugates is slightly less than *Streptococcus* serum antibody, but the ELISA titer of conjugates is 1:1024, the same as *Streptococcus* serum antibody. Therefore, the conjugates do not affect *Streptococcus* serum antibody response efficiency.

3.3.2 Coupling increase *Streptococcus* serum antibody response sensitivity

To analyze the *Streptococcus* serum antibody in the conjugates' response sensitivity, conjugates and *Streptococcus* serum antibody were labeled with colloidal gold. According to Fig 3D, we can see that the conjugates combine with *Streptococcus* cell surfaces when conjugates and *Streptococcus* were mixed for 3 min. After mixing for 7 min, conjugates entered into *Streptococcus* bacteria (Fig. 3F). The *Streptococcus* serum antibody combined with the *Streptococcus* cell surface after being mixed for 4 min and entered into the *Streptococcus* bacteria after 8 min, Therefore, the conjugate response sensitivity is higher than *Streptococcus* serum antibody response sensitivity.

3.3.3 Coupling decrease *Streptococcus* serum antibody immunogenicity

Specific antiserum was generated by immunizing rabbits with either *Streptococcus* serum antibody or the conjugates. *Streptococcus* serum antibody and conjugate immunogenicity were detected by an indirect ELISA method. The results show that the titer of antibody against *Streptococcus* serum antibody is 1:64, and the titer of antibody against conjugates is only 1:8 (Fig. 4). The *Streptococcus* serum antibody immunogenicity is four times the immunogenicity of the conjugates.

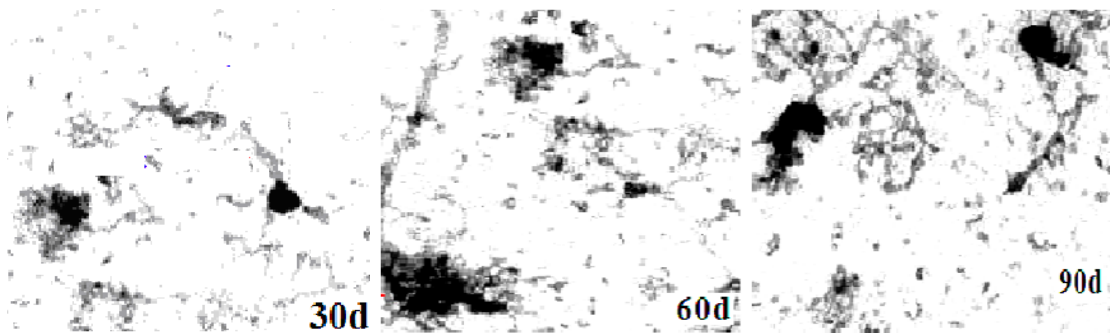


Fig. 1. Scanning Electron Microscopy (SEM) images of the conjugates

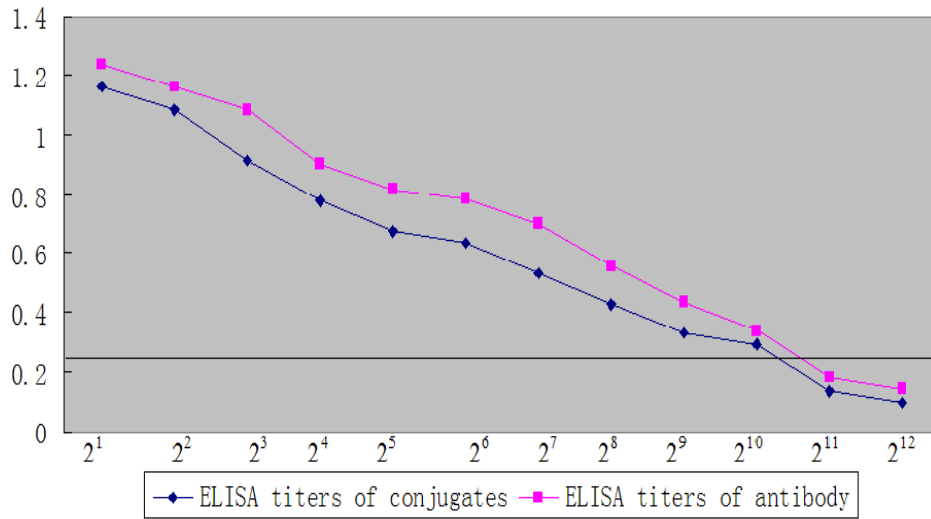


Fig. 2. Conjugates do not affect *Streptococcus* serum antibody response efficiency

3.4 Conjugates Enhance Sulfate Amikacin Biological Activity

3.4.1 Conjugates reduce the acute toxicity of sulfate amikacin

Mice were injected intraperitoneally with sulfate amikacin (125 mg/kg body weight) and conjugates (750 mg/kg body weight). After seven days, five of the mice injected with sulfate amikacin were dead, but no mice injected with the conjugates died. Only 20% of the mice injected with conjugates had reduced ambulation and sleepiness symptoms, but they returned to normal two hours after the injection.

3.4.2 Conjugates improve sulfate amikacin antimicrobial activity

To test sulfate amikacin antimicrobial activity, we measured the MIC and MBC of sulfate amikacin and conjugates. Table 1 data indicate that the

Streptococcus MIC and MBC of conjugates are 0.5 µg/mL and 1 µg/mL, respectively, which are 20 and 50 times greater than sulfate amikacin, respectively. The bacteriostatic effects of *Staphylococcus aureus* and *E. coli* conjugates are not obvious compared with *Streptococcus*.

To further observe curative effects, serum antibodies (0.2 mg), sulfate amikacin (0.2 mg) and conjugates at three concentrations (0.4 mg, 0.2 mg, 0.1 mg) were injected into a *Streptococcus* animal model, respectively. As shown in Table 2, the effective rate and cure rate of conjugates at 0.4 mg is 100% and 90%, respectively, while the rates for sulfate amikacin are 50% and 10%, respectively. Conjugates have twice the effective rate and nine times the cure rate of sulfate amikacin. We also noticed that conjugates can clearly improve sulfate amikacin antimicrobial activity.

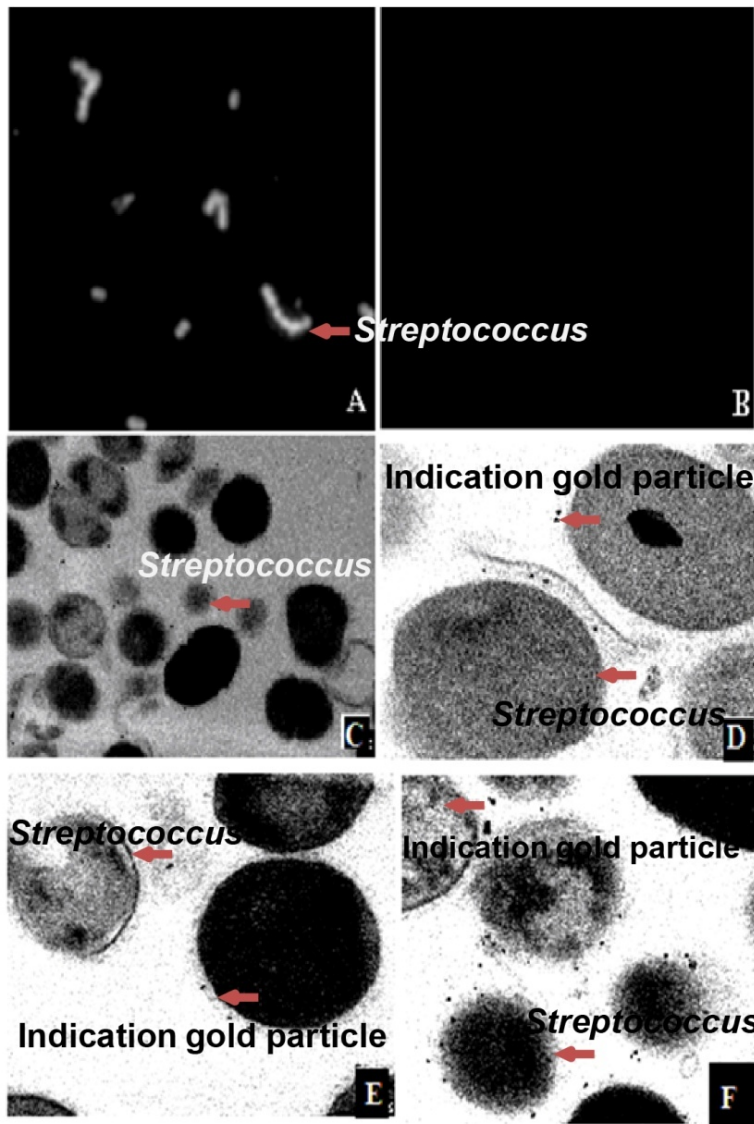


Fig. 3. Conjugates effect on biological activity of *Streptococcus* serum antibodies
 (A) Immunofluorescence microscopy image of the mixture of the conjugates and *Streptococcus* incubated for 3 min (100x). (B) Immunofluorescence microscopy image of the mixture of the conjugates and *Streptococcus* incubated for 2 min (100x). (C) *Streptococcus* without the immunogold-labeled conjugate (50,000x). (D) Immunogold-labeled conjugates and *Streptococcus* incubated for 2 min (50,000x). (E) Immunogold-labeled conjugates and *Streptococcus* incubated for 3 min (50,000x). (F) Immunogold-labeled conjugates and *Streptococcus* incubated for 7 min (50,000x).

3.4.3 Conjugates improve sulfate amikacin bioavailability

To further study the change of sulfate amikacin metabolic parameters, sulfate amikacin and conjugates were injected intraperitoneally (i.p.) into the rabbits and pharmacokinetic parameters were obtained from plasma concentration-time data treated with the MCP-KP pharmacokinetic program. Table 3 data show

that the half-life ($T_{1/2}$) of sulfate amikacin terminal elimination extended, the drug-time area under the curve (AUC) increased, and the apparent volume of distribution (VD) and clearance rate (CL) decreased in conjugates. Pharmacokinetic parameters changed significantly ($P < 0.01$) in conjugates compared with sulfate amikacin.

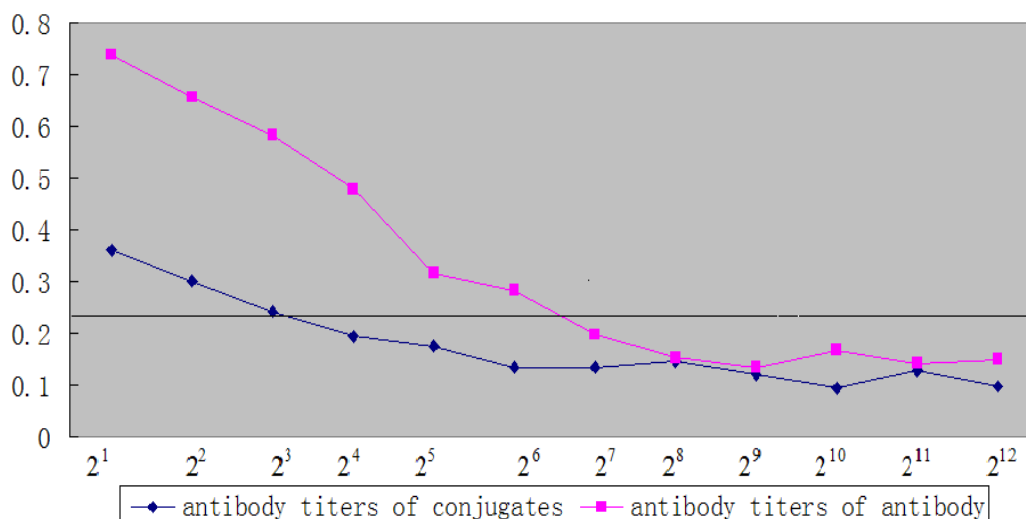


Fig. 4. Conjugates decreased *Streptococcus* serum antibody immunogenicity

4. DISCUSSION

Because the main function of antibody is leading as a role of navigation, and *Streptococcus* has multiple serum type, we prepared rabbit antisera antibody with *Streptococcus01026* strain (Purchased from The Institute of Microbiology, Hunan Province, China), but not monoclonal antibody of *Streptococcus*. Mice and rabbits used in the experiment were all healthy animals, and the blank control was set up in the experiment. The detection results showed that *Streptococcus* serum antibody's specificity which against *Streptococcus* was good.

Streptococcus serum antibodies, sulfate amikacin and PEG6000 were mixed (400:2:9) to form conjugates. Response specificity assays show that conjugates specifically bind *Streptococcus* (Fig. 3A-B). Although mice are particularly sensitive to mouse anti-rabbit xenogenic responses, the terminal proteinuria scores applied to validate the rabbit anti-*Streptococcus* serum antibody and sulfate amikacin conjugates are $<2 \text{ mg } 24 \text{ h}^{-1}$ [15]. The immunological test results indicate that coupling changed *Streptococcus* serum antibody antigenicity. (Fig. 2). At the same time, conjugates increase *Streptococcus* serum antibody response sensitivity (Fig. 3D-F). Conjugates not only improve the antibody targeting but also significantly reduce the body's resistance to antibodies [16]. Therefore, antibodies can be effective as bacteria-targeted drugs.

The increase in the *Streptococcus* serum antibody response sensitivity in conjugates can be mainly attributed to the mechanism of antibody targeting *in vitro* [17]. The negative charge of an antigen decreases when an antibody binds with antigen, which promotes the negative charged antibody to move to the antigen. The speed of antibody moving to the antigen mainly depends on the binding speed of the antigen and antibody and the antibody moving speed in the solution. The faster the binding speed of the antigen and antibody, the greater the voltage difference is around the antigen and stronger the antibody attraction. Because the conjugates are composed of *Streptococcus* serum antibody, PEG6000 and sulfate amikacin, the conjugates have better water solubility than the *Streptococcus* serum antibody, therefore the conjugates move faster than the *Streptococcus* serum antibody in the electrolyte solution. At the same time, PEG6000 is fat-soluble and can stimulate the conjugates passing quickly into the cell membrane. The conjugates entered into *Streptococcus* bacteria faster than the *Streptococcus* serum antibody (Fig. 3D-F).

The response immunogenicity results indicated that *Streptococcus* serum antibody immunogenicity is four times higher than the conjugates (Fig. 4). PEG6000 is a type of coupling agent often used during conjugate formation [18] that allows conjugates to have better water solubility and reduces the conjugate's immunogenicity [19].

To examine sulfate amikacin biological activity, we analyzed the sulfate amikacin acute toxicity, antimicrobial spectra and pharmacodynamics *in vitro* and *in vivo*. Results show that the conjugates reduced the sulfate amikacin acute toxicity, narrowed the antimicrobial spectra and enhanced the pharmacodynamics (Tables 1-3). The polymers of the sulfate amikacin and *Streptococcus* serum antibody are more safe and effective than sulfate amikacin, which will provide a theoretical and experimental basis for bacteria-targeted drug development.

The antibacterial effects of conjugates may be due to adsorption, the release on contact and membrane fusion. First, conjugates attach on the surface of bacterial cells through the serum antibody. Second, the PEG6000 in the

conjugates and bacteria experience a contact release effect and the conjugates enter into the bacterium by increasing the membrane permeability or membrane fusion [20] because PEG6000 is easily dissolved in lipids and induces membrane fusion. Once fusion of the conjugates and bacterial cell membrane occurs, the wall and the membrane of the bacterial cells are damaged, which increases membrane permeability. Finally, the balance of osmotic pressure is broken, and the loss of intracellular material results in cell death [21]. Additionally, the antibody carrying conjugates can directly enter into the cell cytoplasm and exert antibacterial activities. These reasons enable the conjugates to significantly outperform the sulfate amikacin in terms of antibacterial activity.

Table 1. The comparison of *in vitro* antimicrobial activity of sulfate amikacin and conjugates

Strain	Conjugates		Sulfate amikacin	
	MIC	MBC	MIC	MBC
<i>Streptococcus</i>	0.5 µg/ mL	1 µg/mL	10 µg/mL	50 µg/mL
<i>Staphylococcus</i>	>5 mg/ mL	>5 mg/mL	10 µg/mL	50 µg/mL
<i>Escherichia coli</i>	>5 mg/mL	>5 mg/mL	5 µg/mL	10 µg/mL
<i>Salmonella</i>	>5 mg/mL	>5 mg/mL	1 µg/mL	5 µg/mL

Table 2. The comparison of curative effect of sulfate amikacin and conjugates in *Streptococcus* animal model

Category and the total number of treatment	Effective number	Cure number	effective rate (%)	cute rate (%)
Serum antibody 30	0	0	0%	0%
Sulfate amikacin 30	15	3	50%	10%
Conjugate (0.4 mg) 30	30	27	100%	90%
Conjugate (0.2 mg) 30	30	22	100%	75%
Conjugate (0.1 mg) 30	30	18	100%	65%

Table 3 The comparison of pharmacokinetics parameters of sulfate amikacin and conjugates

	T1/2(h)		Vd(L/kg)		CL(L/kg.h)		AUC(mg.h/L)	
	I	II	I	II	I	II	I	II
1	0.986	3.9564	0.290	0.0524	0.1882	0.0092	53.12	204.74
2	0.962	4.0576	0.161	0.0533	0.1158	0.0091	86.33	206.55
3	0.771	3.6430	0.229	0.0543	0.204	0.0103	48.99	181.81
4	0.884	3.7814	0.205	0.0542	0.1604	0.0100	62.33	181.89
5	1.073	3.7200	0.137	0.0549	0.0884	0.0102	113.09	183.77
6	1.317	3.6430	0.218	0.0543	0.1057	0.0103	94.61	181.81
7	1.249	3.8502	0.340	0.0536	0.1741	0.0101	57.45	202.34
8	1.457	3.9319	0.290	0.0537	0.1273	0.0099	78.57	195.72
9	1.437	3.8134	0.304	0.0539	0.1354	0.0097	73.86	187.78
10	1.045	3.7313	0.169	0.0540	0.1121	0.0095	89.20	180.69
\bar{X}	1.12	3.8316	0.23	0.0538	0.14	0.0098	76	191.75
±s	0.24	0.1712	0.07	0.001	0.04	0.0006	20	12.72
P	<0.01		<0.01		<0.01		<0.01	

I; sulfate amikacinII; conjugates

This animal model is mice systemic infection animal model. Since *Streptococcus*₀₁₀₂₆ strain in *Streptococcus* challenge experiment is Pathogenic bacteria from swine, it is difficult to make a local infection model of mice. The results of *Streptococcus* challenge experiment showed that the effective rate and cure rate of conjugates at 0.4 mg is 100% and 90%, respectively, while the rates for sulfate amikacin are 50% and 10%, respectively, so the conjugate form is better than the free drug in protection of systemic infection.

Sulfate amikacin was protected by PEG6000 and *Streptococcus* serum antibody through coupling. The retention time of was longer, and the amount of sulfate amikacin removed was less than these before coupling. At the same time, conjugates can target pathogens by antibody binding [22]. These reasons result in the increase in the drug-time area under the curve (AUC) and bioavailability.

The application of antibody-based drugs for targeting bacteria is beneficial to humans and animals. The diagnosis of bacterial disease can not only be qualitative but also quantitative [23]. According to the number of the pathogenic bacteria in the body, bacterial diseases may be cured by antibody-targeted drugs.

5. CONCLUSION

The results indicated that coupling reduced the acute toxicity of sulfate amikacin, improved sulfate amikacin bioavailability and antimicrobial activity of sulfate amikacin. Antibody-targeted drugs can eliminate the adverse reactions and organ damage caused by drug residues, reduce or stop the formation the drug resistant strains, prolong the life of antibiotics and give obsolete antibacterial drug new life. The combination effect on the antibacterial activity of drug and the biological activity of serum antibody is helpful for the practical application of targeted drugs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal experiments were performed following a protocol approved by the Institutional Animal Committee of Hunan Agricultural University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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