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Biological Safety Assessment of the Silk Fibroin-Based Nerve Guidance Conduits in Vitro and in Vivo

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Abstract. Silk fibroin (SF) has been used extensively in the biomedical field. We have previously reported that we have developed and evaluated a novel biomimetic design of the silk fibroin-based nerve guidance conduit (SF-NGC) used for peripheral nerve regeneration. In the present study, we have evaluated the biological safety of the SF-NGC according to the ISO 10993. For assessment of the SF-NGC, the acute toxicity test was performed; Ames test was used to evaluate genotoxicity in vitro, bone marrow micronucleus test was reckoned to identify genotoxicity in vivo; teratosperm ratio assay and morphological observation of the testis histology were carried out to estimate the toxicity on reproductive system in mice; and subcutaneous implant test in rabbits was used to investigate the local effect after implantation. No significant changes in body weight, behavior, mortality were detected in the acute toxicity test. Moreover, neither Ames test nor micronucleus assay results indicated that SF-NGC have genotoxic potency. Based on the results of the teratosperm ratio assay and morphological observation of the testis, SF-NGC showed no toxicity effects on reproductive system in mice. The results of subcutaneous implant test in rabbits indicated that the SF-NGC had a good compatibility with the surrounding tissues. The data obtained in this paper provides powerful support for SF-NGC application in nerve regeneration study.

Keywords: acute toxicity; genotoxicity; reproductive toxicity; silk fibroin; subcutaneous implantation

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1. Introduction

Raw silk from the silkworm, *Bombyx mori*, has been used as biomedical suture material for centuries due to their impressive mechanical properties and flexibility.[1-4] However, some biological responses to the protein have raised questions about biocompatibility during decades of use.[5] The identification of contaminating sericin as the source of immunological problems sparks new interest in silk fibroin (SF) as a native biomaterial. The silk fibroin protein is biocompatible, degrades slowly in the body, is readily modified into a variety of formats and generates mechanically robust materials.[6] In addition, it has several attractive properties, including compliance, variable size, good oxygen and water vapor permeability.[7] These characteristics of SF provided important clinical repair options for many applications. In decades, the purified SF has become a novel, promising biomaterial and found rapidly increasing applications in biomedical fields including tissue engineering of bone, cartilage, tendon, skin, ligament, and blood vessels.[8-13] We have also studied on the utility of SF for nerve tissue engineering.[14-16]

Peripheral nerve repair remains a common but challenging clinical problem. Tissue-engineered nerve guides have been developed as a promising option for nerve regeneration, due to their mechanical support and chemical stimulation for axonal elongation.[17] Especially for larger nerve defects or gaps, implantation of a nerve graft is often necessary to bridge the proximal and distal nerve stumps for facilitating nerve regeneration and functional recovery. Processing methods developed in our laboratory allow for the manufacture of silk fibroin-based nerve guidance conduit (SF-NGC) of varying inner diameter, porosity, and mechanical strength.[14] We have reported on good in vitro and in vivo biocompatibility of SF-NGCs with nerve tissues and cells.[16] But according to the ISO 10993, a qualified medical device should do a series of biology evaluations prior to a clinical study. The evaluation tests usually include of the tests for acute toxicity, cytotoxicity in vitro, genotoxicity, toxicological effects on reproductive system and local effects after implantation. The cytotoxicity of the SF-NGCs in vitro has been assessed using an internationally accepted method from the ISO 10993-5 as previously reported.[16]

The present study was performed to evaluate the acute toxicity, genotoxicity, toxicological effects on reproductive system and local effects after implantation of the SF-NGCs. According to the practice ASTM F 750 (Method B for intraperitoneal injection) which was recommended by the ISO 10993-11, the acute toxicity of the SF-NGCs was evaluated. To confirm the genotoxicity of the SF-NGCs, Ames test and micronucleus assay were performed. To determine the toxicological effects of the SF-NGCs on reproductive system in male mice, the sperm reserves in epididymidis were measured and histopathological lesions in the seminiferous tubules were

observed. To assess the local effects of the SF-NGCs after implantation, the SF-NGCs were subcutaneously implanted in rabbits, and the general observation and histological examination were performed at 4, 8, 12, and 24 weeks after the implantation.

2. Materials and Methods

2.1 Preparation of SF-NGCs

Raw silk fibers (from *Bombyx mori* cocoons) were bought from Xinyuan Sericulture Company (Haian, Jiangsu, China). The sericin coating of silk fibers was removed via degumming process of boiling in aqueous Na_2CO_3 solution as previously described.[18] Degummed SF fibers were first dissolved in a tertiary solvent system of $CaCl_2/H_2O$/EtOH solution (mole ratio 1:8:2) at 80˚C for 1 h, and then dialyzed against distilled water in a cellulose tube (molecular cutoff = 12,000-14,000) at room temperature for 3 days. The resulting SF aqueous solution was concentrated with a rotary evaporator under vacuum at 40˚C and ready for use.

SF-NGCs were fabricated according to our developed procedures as described previously.[14] In brief, a stainless-steel casting mold was used to fabricate a tubular structure. The mold consisted of an inner pillar and an outer tube, both of which were fixed on the mold bottom, determining the diameter and wall thickness of conduits. SF fibers were enwound evenly around the inner pillar of the mold before the SF aqueous solution was injected into the mold. Then demolding under lyophilization was performed in a stepwise manner to furnish SF-NGCs, which were further treated in 80% (v/v) methanol solution to achieve insolubility of SF in water.

2.2 Preparation of SF-NGCs extract liquid

The SF-NGCs, as the extract phase, were placed in 0.9% normal saline which served as the extract medium. According to the ISO10993-12: 2002, 120 cm^2 of extract phase surface area in 10 ml of extract medium was selected as high dose for the relational tests, while 60 cm^2/10 ml and 30 cm^2/10 ml were selected as middle and low dose levels respectively. The extraction was allowed to proceed at 37°C for 72 ± 0.5h, and sterilized prior to use.[15,16]

2.3 Animals

The mice were housed 5 per IVC (individually ventilated cages), and were maintained under a controlled environment with temperature at 23 ± 2°C, relative humidity at 55 ± 5%, and a 12 h/12 h light/dark cycle throughout the experiment. All

experimental procedures involving animals were conducted as per Institutional Animal Care guidelines and approved ethically by the Administration committee of experimental animals, Jiangsu Province, China.

2.4 Acute toxicity test in mice

Acute toxicity test is a way designed to detect the presence of injurious leachable substances.[19] In the present study, animals were divided into two groups (a blank and a sample). Ten ICR mice, five male and five female, aged 45-50 days and weighing 18-22 g, were used for each group. The extract liquids of SF-NGCs were prepared by 120 cm^2 of extract phase surface area in 10 ml of 0.9% normal saline. The dose of the extract liquids was 50 ml/kg of body weight for each mouse, injected at a steady rate of not more than 0.1 ml/s. The animals were administered normal saline and the SF-NGCs extract liquids two times in 8 h. The animals were observed immediately post-injection, again 4 h later, and then not earlier than 24, 48, and 72 h, respectively, post-injection for symptoms of normal, slight, moderate, or marked toxicity or death according to the Table 1. The body weights of all animals were recorded at 24, 48, and 72 h post-injection.

2.5 Genetic toxicity study

2.5.1 Ames test

Ames test was performed to evaluate the mutagenicity of SF-NGCs in a bacterial reverse mutation system.[21] Briefly, the strains of Salmonella typhimurium (TA97, TA98, TA100 and TA102) were grown overnight in the nutrient broth in a shaking incubator at 37°C in the presence or absence of a rat S9 metabolic activation system. The SF-NGCs extract liquids were prepared by 60 cm^2 of extract phase surface area in 10 ml of 0.9% normal saline. 2-aminofluorene at a concentration of 10 μg/plate for TA 97, TA98 and TA100 strains, 1, 8-dihydroxyanthraquinone at a concentration of 50 μg/plate for TA102 strain, 9-aminoacridine at a concentration of 0.2 μg/plate for TA97 and TA98 strains and MMS at a concentration of 10 μg/plate for TA100 and TA102 strains were used for positive controls.

The microsomal fractions for metabolic activation were prepared from the livers of adult male Sprague-Dawley rats. The animals were sacrificed at 5th day after a single administration of Aroclor 1254 (500 mg/kg) by intraperitoneal injection. 25 % of liver homogenate prepared in 0.15 M KCl was centrifuged for 10 min at 9,000 rpm. The supernatant fraction (S9) was stored at −80°C until use. The composition of S9 mixture was prepared as follows: 0.1 M phosphate buffer, pH 7.4, 4 mM NADP, 5 mM glucose-6-phosphate, 30 mM $MgCl_2$, 8 mM KCl salt solution, and 4% of S9 fraction. Each of fresh bacterial suspension, 0.1 ml of S9 mixture (or 0.5 ml phosphate buffer), and 0.1 ml of the test substance were mixed in each tube. After vigorous

shaking on a shaking incubator, 2 ml of liquid top agar was added to each tube, and the mixture was poured onto the agar plates. The plates were incubated at 37°C for 48 h until counting the revertants. An increase by a factor of 2 fold above the negative control level was taken as an indication of a mutagenic effect.

2.5.2 In vivo micronucleus assay

The ICR mice, aged 45-50 days and weighing 18-22 g, were randomly divided into five groups of 10 animals (5 male and 5 female) each: a high dose group, a middle dose group, a low dose group, a negative control group and a positive control group. Administration doses were determined based on the Standard Practice ASTM F 750 standard. The intraperitoneal injection dose of the SF-NGCs extract liquids is 50 ml/kg of body weight for each mouse. 40 mg cyclophosphamide/kg of body weight was selected as a positive control group dose, while 0.9% normal saline was used as a negative control. Animals were administered normal saline, cyclophosphamide, or various doses of the SF-NGCs extract liquids four times at an interval of 24 h by intraperitoneal injection. All animals were sacrificed by cervical vertebra dislocation at 24 h after the last intraperitoneal injection. Both femora were taken out and the muscle tissue around bones was cleaned out. The proximal ends of the femora were opened and the bone marrow was flushed into a 5 ml centrifuge tube by 3 ml fetal bovine serum. The mixture was well mixed and then centrifuged for 5 min at 1,000 rpm. The most of supernatant was discarded. Following mixing, a drop of the sediment was smeared onto a clean slide and air-dried. The dried slides were fixed by immersion in 95% methanol for 15 min and stained in ordinary staining jars with Giemsa Working Solution for 15 min. Stained slides were washed with distilled water slowly and air-dried, then analyzed under light microscopy. All slides were coded to ensure that the evaluation was blinded. To determine the presence of micronuclei in bone marrow cells of mice, 1,000 polychromatics (PCEs) were analyzed per animal.

Both biological and statistical significance were considered for evaluation of a positive response. The test substance was considered positive for a mutagenic response if there was a statistically significant in the number of micronucleated PCEs at any dose of the SF-NGCs extract liquid, compared to the concurrent negative control group.

2.6 Toxicity test on reproductive system

The Male ICR mice, aged 45-50 days and weighing 18-22 g, were randomly divided into five groups of 5 animals each: a high dose group, a middle dose group, a low dose group, a negative control group and a positive control group.

The average body weights set for five groups were almost the same right before the first administration. The intraperitoneal injection dose of the SF-NGCs extract liquids was 50 ml/kg of body weight for each mouse. The cyclophosphamide (40

mg/kg) was used as a positive control, and 0.9% normal saline served as a negative control. Animals were administered normal saline, cyclophosphamide, or various doses of SF-NGCs extract liquids every day by intraperitoneal injection for five days all. The animals were sacrificed by cervical vertebra dislocation after 35 days of the first administration. Epididymides and testes were isolated and cleared of adhering tissues. Then epididymides were cut into small pieces with ophthalmologic scissors, and homogenized for 1 min in 3.0 ml of physiological saline solution. The homogenate was filtered through 4 layers microscope lens paper. Filtrate was centrifuged for 5 min at 1,000 rpm. Most of the supernatant was removed. The remaining supernatant was well mixed with the precipitate, and then the homogenized mixture was smeared on clean slides. Slides were dried at room temperature, followed by fixing in 95% methanol for 5 min and stained with 0.5% eosin ethanol solution. Stained slides were rinsed by 95% ethanol solution, and examined under a light microscope. For assessing the morphological abnormalities, at least 1000 sperm cells were tested per mouse. The percentage of abnormal sperms was recorded.

The testis tissue was trimmed and fixed in buffered 4% paraformaldehyde overnight at 4°C, embedded and cut on a cryostat into 8 μm thick sections that were perpendicular to the longest axis of the testis, followed by H&E staining and observation under light microscopy.

2.7 Subcutaneous implant test

SF-NGCs were each sheared into a 10-mm-long one, which were dried in an oven at 60°C to a constant weight and sterilized prior to use. New Zealand adult rabbits were used as an animal model for investigating the local effects after they were subcutaneously implanted. The pre-numbered rabbits were positioned in ventral recumbence, and their central backs (size: 8×14 cm^2) were depilated and sterilized for surgery. After the animal was anaesthetized by ear vein injection of 3% sodium pentobarbital (1.2 ml/kg), 6 skin incisions (each approximately 5 mm long) were created on its central back, and the fascia underlying the incisions was split, followed by implantation vertically with the SF-NGC. The 6 implantation sites were equally located on the two sides around the spinal column with each 2 sites separated by a 4-cm distance, and 6 SF-NGCs were separately implanted on either side. Then the surgical incisions were closed in a routine manner. After surgery, all animals were housed and fed routinely, and monitored for the changes in their general conditions.

The subcutaneous implantation incisions of rabbits were re-opened at 4, 8, 12, and 24 weeks after implantation, respectively, for gross observation, and the implanted SF-NGCs were then taken out. The harvested SF-NGCs were fixed in buffered 4% paraformaldehyde overnight at 4°C, embedded, and cut on a cryostat into 10-μm-thick sections that were perpendicular to the longest axis of the SF-NGCs, followed by haematoxylin and eosin (H&E) staining and observation under light microscopy. At 24 weeks, we were unable to retrieve conduits due to fragmentation

because of the degradation.

2.8 Statistical analysis

The data were expressed as means ± S.E.M. and analyzed by multiple t tests or one-way ANOVA and subsequent Scheffe's post hoc test using a SPSS 13.0 software package (SPSS Inc., Chicago, IL). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Acute toxicity in mice

There was no clinical signs, adverse effects, abnormal behavior or mortality was observed on tested animals of the SF-NGCs extract liquids group. The body weights of all animals at 24, 48, and 72 h post-injection were shown in Figure 1. The SF-NGCs extract liquids group showed no remarked alteration in their body weights at 24, 48, and 72 h post-administrations as compared to the negative group. And the body weights of mice in sample group continuous increased in the 72 h post-injection. So, the response after injection of the SF-NGCs extract liquids was normal according to the Table 1.

3.2 Genotoxicity

Table 2 showed numbers of Salmonella typhimurium revertants induced by SF-NGC with or without metabolic activation. The SF-NGCs extract liquids at any dose did not produce any mutagenic responses in the absence or presence of S9 on the Salmonella typhimurium strains TA97, TA98, TA100 and TA102.

Figure 2 showed the results of the micronucleus test. The number of naturally-occurred micronucleus was less than 2 out of 1,000 PCEs. Treatment of mice with the SF-NGCs extract liquids did not elevate frequency of micronucleated PCEs at any dose. So, SF-NGC did not induce micronuclei in bone marrow cells of mice. However, treatment with CP 40 mg/kg BW, a known mutagen, led to a significant elevated frequency ($P < 0.01$) of micronucleated PCEs.

3.3 Toxicity on reproductive system

Incidences of morphological abnormalities in sperm from mice treated with the SF-NGCs extract liquids are summarized in Figure 3. The number of naturally occurred teratosperm was less than 20 out of 1,000 sperms. Even the highest dose of the SF-NGCs extract liquids was not induced sperm aberration in epididymidis of the

mice. In comparison to the negative control group, the incidence of abnormally shaped sperm cells was significant increased in positive control group which were given cyclophosphamine (CP) 40 mg/kg BW.

All mice in the negative and SF-NGCs groups showed normal histological pattern in the testis (Figure 4A, C, D, E), whereas mice treated with 40 mg/kg BW cyclophosphamine (CP) showed histopathological changes in the seminiferous tubules. In addition to degeneration of germ cells in atrophied seminiferous tubules, the lumen of the seminiferous tubules was enlarged (Figure 5B).

3.4 Local effects of SF-NGCs after implantation

During the 6 months, the wounds were free from suppuration and necrosis after subcutaneous implantation in all periods. The implants were tolerated well by the host animals and no abnormal conditions were observed in rabbits based on gross analysis. There were no obvious redness and edema, and the formation of fiber lumps at the implantation site. The SF-NGCs degraded gradually and it was hardly to see or retrieve the SF-NGCs at 24 weeks (Figure 5).

Figure 6 and Figure 7 show the photomicrographs of the tissues implanted with SF-NGCs at different time points. At 4 weeks after implantation, connective tissue encapsulations were seen around the implantations (Figure 6A). Acute inflammatory reaction with many neutrophils infiltration, a few lymphocytes, macrophages and phagocytic cells were observed on the tissues around the implants (Figure 7A). At 8 weeks after implantation, the degree of inflammatory reaction was much milder than those at 4 weeks and neutrophils infiltration were significant alleviated, but the number of lymphocytes and phagocytic cells was slightly increased (Figure 7B). Heavy tissue and vascular ingrowth occurred at two ends of the conduit. And the conduit walls were looser than those retrieved at 4 weeks (Figure 6B). At 12 -week postoperatively, the degree of inflammatory reaction for the tissues implanted with SF-NGCs was much less than those retrieved at 4-week and 8-week post-implantation. Very few lymphocytes and neutrophils still existed in surrounding tissues of the conduits remained, but the number of phagocytic cells was significantly increased (Figure 7C). The conduits walls were much looser. The lumen of the conduit was full of regenerated tissue and vascular (Figure 6C). At 24 weeks, neither the inflammation reaction nor the SF-NGC was observed due to the biodegradation. In general, it can be seen that the inflammatory reaction was gradually lighted with the time prolonged and disappeared in the end. The observations reflected good tissue compatibility between the SF-NGCs and the surrounding tissues.

4. Discussions and Conclusions

There is a continuous search for SF-NGC that may serve the purpose of peripheral nerve regeneration tissue engineering applications. A significant issue to consider regarding the use of any biomaterial is biological safety. We have previously reported that good biocompatibility of SF or their extraction fluids with peripheral nerve tissues or cells.[15] We also have demonstrated that the SF-NGCs were quit biocompatible with the surrounding tissues or cells and generally suitable to a certain degree for bridging peripheral nerve defects.[16] But according to the International Standards ISO 10993, a qualified medical device should make relevant biology evaluation prior to a clinical study.

So a series of biology evaluations were carried out in the present study. Acute toxicity of the SF-NGCs was assessed using an internationally accepted method from the Standard Practice ASTM F 750 (Method B for intraperitoneal injection). No toxic signs or mortality were noted. The body weights were not affected by administration of the SF-NGCs extract liquids suggesting that SF-NGCs had no deleterious effects on health status, growth or development of the animals.

Ames test and micronucleus assay were performed to evaluate the genetic toxicity of the SF-NGCs. Neither mutagenic effects nor abnormal micronucleus formation were detected indicating that SF-NGCs produced no genetic toxicity on mice. Moreover, toxicological effects on reproductive system of SF-NGC were assessed in mice. No significant alteration on morphologically abnormal spermatozoa and testis was observed suggesting that there is no genetic damage on male reproductive organs by administration of SF-NGC extract liquids.

Subcutaneous implant test with the SF-NGCs was executed to estimate the local effects of the SF-NGCs after implantation. After subcutaneous implantation, the wounds were free from suppuration and necrosis in all periods. With the time prolonged, the inflammatory reaction is gradually lighted and disappeared in the end, which accorded with the previous report on the in vivo foreign body response.[21] It was likely that the continuously increased phagocytic cells at the first 12 weeks were due to the gradually degradation of the SF-NGCs. At 24-week, the SF-NGCs were almost completely degraded by the host tissues without the formation of any fiber capsule.

In summary, the resulted data include the acute toxicity, genotoxicity, toxicological effects on reproductive system and local effects after implantation of SF-NGCs. No effect was observed in any of the parameters measured suggesting the absence of a treatment-related adverse effect of the SF-NGCs extract liquids. The data support the safety of SF-NGCs for using as nerve graft in nerve regeneration. Therefore, the SF-NGCs which were prepared by our procedure posses with high biological safety had a good potential as medical device for nerve regeneration.

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Response	Description
Normal, no symptoms	Mouse exhibits no adverse physical symptoms after injection.
Slight	Mouse exhibits slight but noticeable symptoms of hypokinesia, dyspnea, or abdominal irritation after injection.
Moderate	Mouse exhibits definite evidence of abdominal irritation, dyspnea, hypokinesia, ptosis, or diarrhea after injection. (Weight usually drops to between 15 and 17 g.)
Marked	Mouse exhibits prostration, cyanosis, tremors, or severe symptoms of abdominal irritation, diarrhea, ptosis, or dyspnea after injection. (Extreme weight loss; weight usually less than 15 g.)
Dead, expired	Mouse dies after injection.

Table 1 Response to systemic injection assay

Group	Number of revertants (Number of colonies/plate)							
	TA97		TA98		TA100		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
SF-NGC	81±5.6	84±11.5	21±2	21±2.6	173±9.1	181±13.5	215±8.5	209±18.8
N. control	94±8.0	94±8.1	19±1.5	24±3.2	175±9.6	182±9.8	256±16.8	262±19.7
P. controls (μg /plate)								
2-AF(10)		634±77.0		2507±431.0		856±112.7		
1,8-DA(50)								826±179.5
9-AA(0.2)	414±78.1		2256±381.0					
MMS(10)					937±75.6		793±150.1	

Table 2 Mutagenic activity of SF-NGCs on Salmanella typhimurium TA98, TA97, TA100, TA102 with or without S9.

Each value shows the means ± S.E.M of number of revertents (n = 3 plates).
Asterisk indicate significant differences from NS (the negative group) **p < 0.01
N, negative; P, positive; AF, aminofluorene; DA, dihydroxyanthraquinone; AA, aminoacridine.

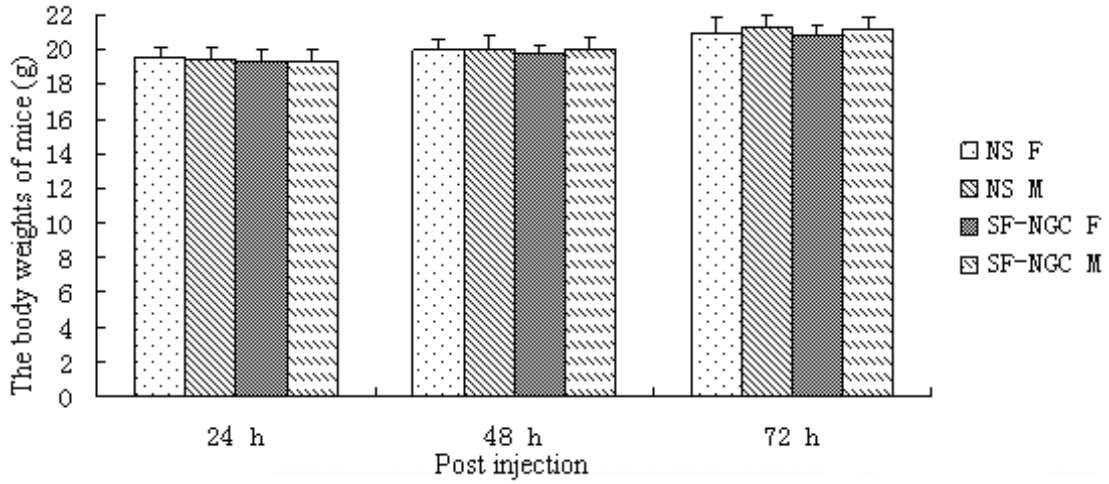

Figure 1. Effect of SF-NGCs on the body weight. Each value shows the means ± S.E.M of body weight (n = 5). No statistical differences were found.

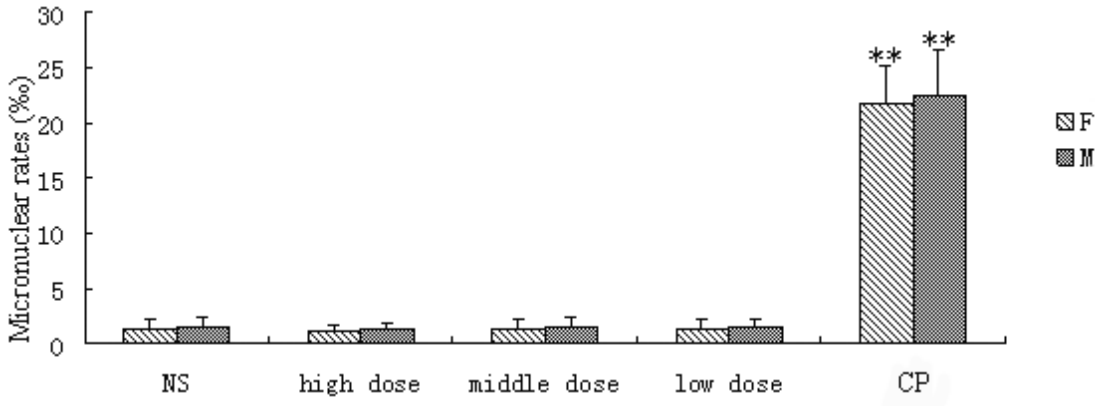

Figure 2. Micronuclear rates with bone marrow cells of mice. Each value shows the means ± S.E.M of micronuclear rates (n = 5). Asterisk indicate significant differences from NS (the negative group), **p < 0.01.
NS: normal saline, CP: cyclophosphamide, F: female, M: male.

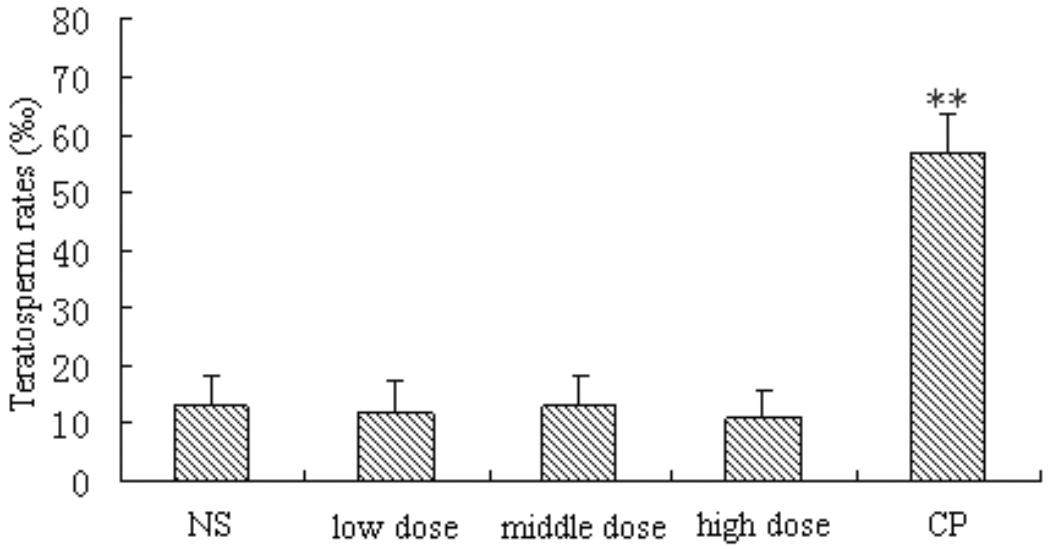

Figure 3. Teratosperm rates in epididymidis. Each value shows the means ± S.E.M of teratosperm rates (n = 5). Asterisk indicate significant differences from NS (the negative group), **$p < 0.01$.

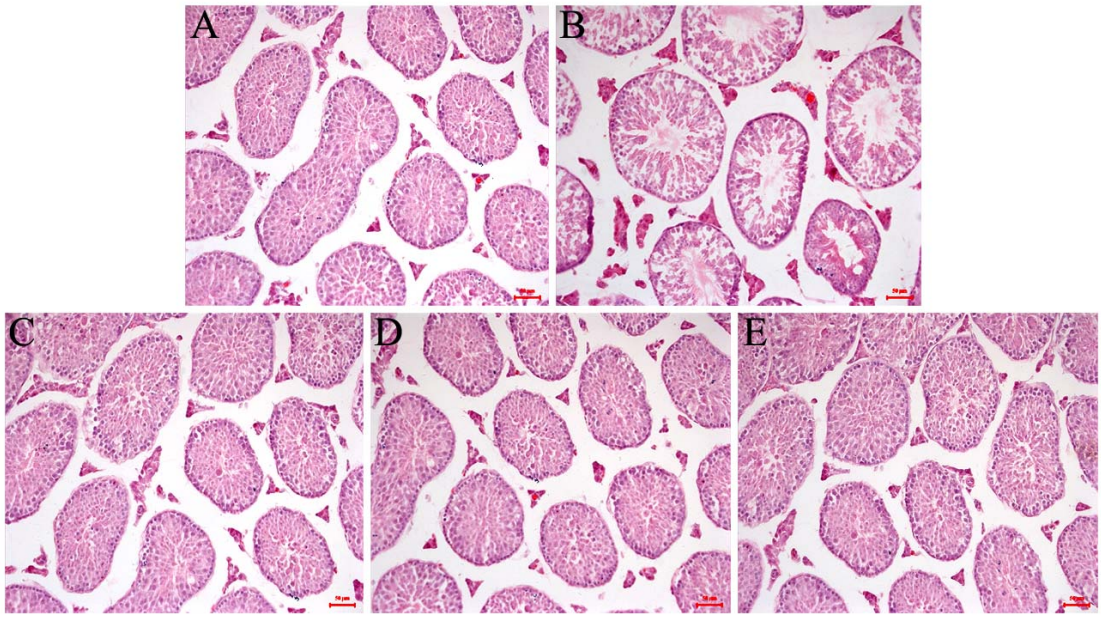

Figure 4. Histopathological of testes. Testes were isolated from the negative control mouse (A), the positive control mouse (B), the low dose treated mouse (C), the middle dose treated mouse (D) and the high dose treated mouse (E). Haematoxylin and Eosin staining, Scale bar = 50μm.

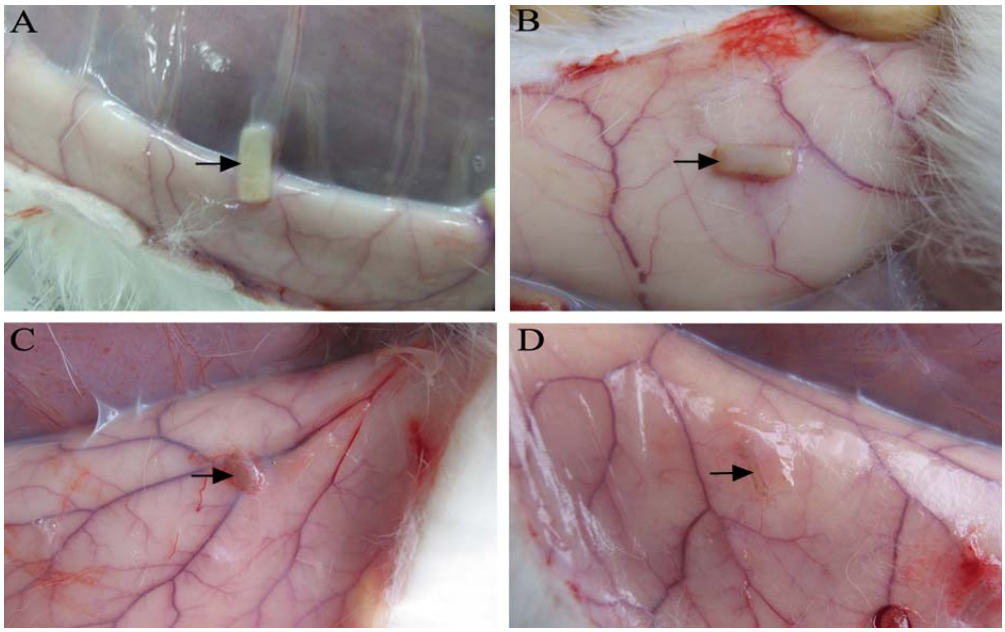

Figure 5. Gross observation of the SF-NGCs post-implantation in vivo at 4-week (A), 8-week (B), 12-week (C) and 24-week (D). Tailed arrows = remaining SF-NGCs.

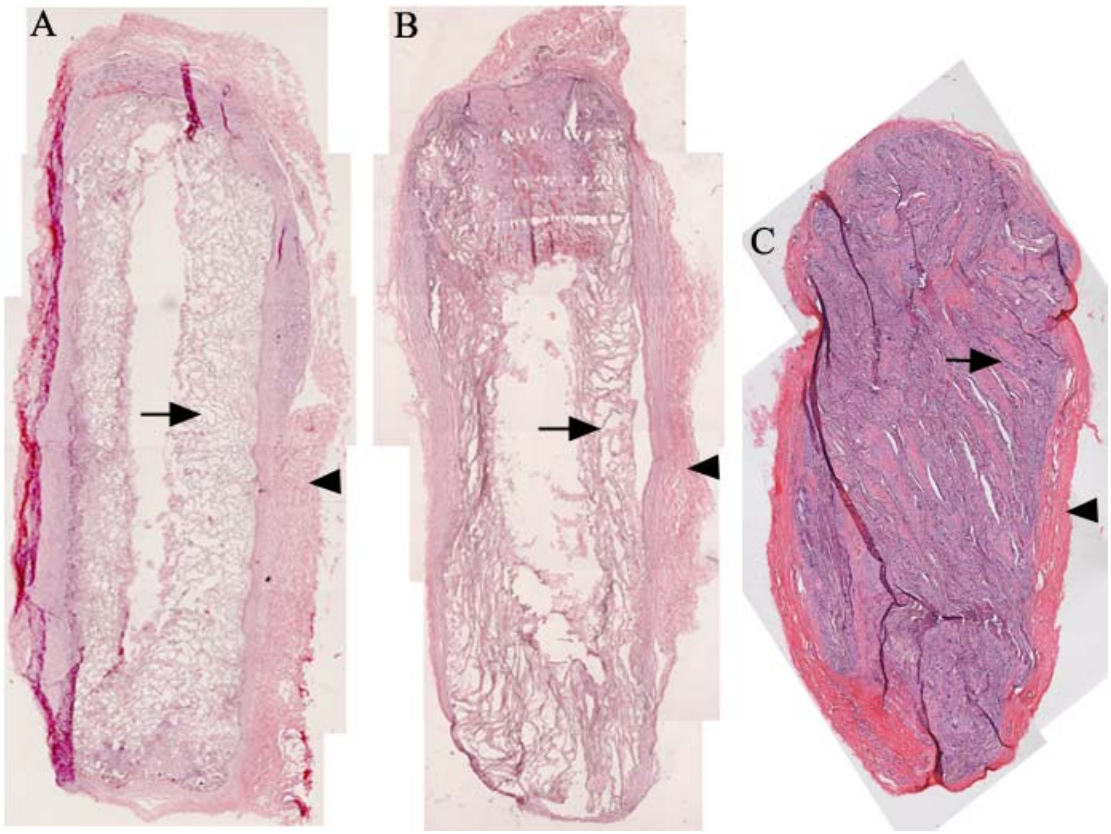

Figure 6. Histopathological of the longitudinal sectioned SF-NGCs obtained at 4-week (A), 8-week (B), 12-week (C), Haematoxylin and Eosin staining, Scale bar=100μm. Tailed arrows = remaining implantation graft. Tailless arrows = connective tissue.

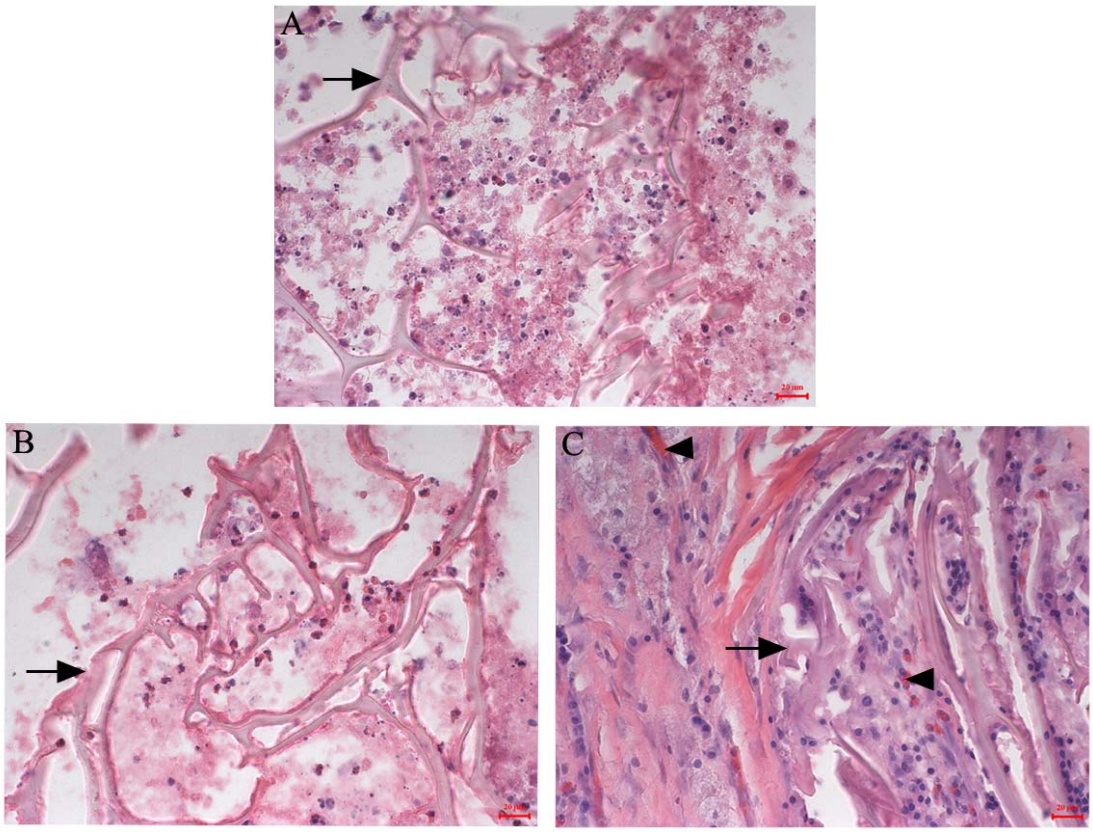

Figure 7. A, B and C are local magnifications of Figure 6A, B and C, repectively, Haematoxylin and Eosin staining, Scale bar = 20μm. Tailed arrows = remaining implantation graft. Tailless arrows = regenerated vascular.

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