ORIGINAL RESEARCH

Effect of *Cissus quadrangularis* Hydrogel on Enhancing Osseointegration of Titanium Implant to Bone: An *In Vivo* Study

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Abstract

Aim: The aim of the study was to evaluate the osteogenic potential of *Cissus quadrangularis* (CQ) hydrogel in enhancing the osseointegration of titanium to the bone in an experimental rabbit model.

Materials and methods: Six adult male New Zealand white rabbits were used in this study. A total of 24 implants (12 coated test implants and 12 uncoated control implants) were placed in these 6 rabbits. A polyethylene glycol (PEG) hydrogel was prepared with the *C. quadrangularis* hydrogel in which the test implants were coated. Each rabbit was operated on both hind legs and one implant, each, was placed in the femur and tibia. Hence, one rabbit received four implants [two test implants (HG coated) and two control implants (uncoated)]. The animals were sacrificed after 4 weeks, and the specimens were histomorphometrically analyzed. The bone-to-implant contact (BIC) and the bone area fraction occupancy (BAFO) were calculated using Image J analysis.

Results: The statistically analyzed values which were obtained by paired *t*-test, revealed that the average mean values were higher in the test implants (coated) than the control implants (uncoated). The BIC values of the test implants were not significantly different from the control implants in the case of both femur and tibia ($p > 0.05$). The test implants showed significantly increased BAFO values in femur ($p < 0.05$). However, the BAFO values of test implants in tibia did not vary significantly from the control implants.

Conclusion: Based on the findings of the study, the authors conclude that the coating of *C. quadrangularis* hydrogel enhances the osseointegration of titanium implants to bone. The further studies need to be designed to check the osseointegrative potential of *C. quadrangularis*.

Clinical significance: The findings of this study suggest that the *C. quadrangularis* hydrogel is a potent osteogenic material that can reduce the osseointegration period and thus enhance the patient compliance toward implant treatment.

Keywords: *Cissus quadrangularis*, Hydrogel, Osseointegration.

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Introduction

Titanium implants have been the best first choice treatment for the replacement of missing tooth/teeth.[1] Many long-term studies have proved titanium implants to be a safe and reliable option for tooth/teeth replacement.[2,3] The treatment times for conventional loading and final prosthesis may take 3–4 months depending on the jaw. Research has been going on in the field of implant dentistry to try and reduce the healing time and waiting period and give as predictable and stable results as conventional loading.

Many modifications to the surface of the implant, both macro and micro, have been researched to enhance the osseointegration of titanium to the bone. Thread geometry variations and incorporation of microrough surfaces have been tried and tested. The implant surface modifications have been the most sought-after area of research for increasing osseointegration and reducing the treatment time. Hicklin et al. placed hydrophilic implants and loaded early in a clinical trial, and their findings assured them to conclude that hydrophilic implants can be used for early functional loading.[4] Wettability or hydrophilicity of the implant surface has shown that the osseointegration is faster and stability of the implants is maintained. The hydrophilic surfaces are already clinically proven in this regard and are available in the commercial market to reduce the waiting period for osseointegration.

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The implant surface has been biofunctionalized in addition to these surface modifications to bring about desirable and predictable results over the conventional surfaced implants. One of the recently documented methods for this biofunctionalization of implants is the use of hydrogels.[5] Hydrogels have been widely used in biomedical applications primarily for the therapeutic potential for wound healing. Hydrogels are being used in conjunction with various components to provide a beneficial environment for wound healing.[6] The studies have reported positive results with the use of hydrogel surface coating of titanium implants. Hydrogels act as

a scaffold to carry various biomimetic molecules to the implant surface so that the process of osseointegration is hastened.[7] Various substances have been used in this context such as bone morphogenic protein-2 (BMP),[8] beta-tricalcium phosphate (bTCP),[9] phosphonic acid, hydroxyapatites, and more. The substances used as hydrogels are poly-ethyl glycols, chitosan, and various other permutations that have been used as scaffolds.

C. quadrangularis is an herb found in the dry and hotter parts of the Indian subcontinent and parts of Africa. It is also known as the Veldt grape. The taxonomy is as follows:

- Kingdom: Plantae
- Subkingdom: Viridiplantae
- Division: Tacheophyte
- Class: Magnoliopsida
- Order: Vitals
- Family: Vitaceae
- Genus: *Cissus*
- Species: *C. quadrangularis* Linn

It finds a special mention in the texts of Indian alternative medicine called Ayurveda for its fracture-setting properties. Ayurveda, the ancient Indian form of medicine, describes it as a *hadjod* or bone setter used for treating fractures. Many modern-day studies validate the use of *C. quadrangularis* as a potent anti-osteoporotic drug.[10,11] Bhat and Chowdhary showed the osteogenic potential of the *C. quadrangularis* extract *in vitro*.[12]

C. quadrangularis has lately been applied in dentistry as well mainly for mandibular fracture healing.[13] Researchers have found that the administration of *C. quadrangularis* had a shorter healing time and there was a reduction in pain and swelling and an elevated serum alkaline phosphatase activity indicating bone regeneration activity.[14–16] Jain et al. have explored *C. quadrangularis* for periodontal bone defect correction.[17,18] It has been researched to be used as a bioactive coating for orthopedic implants.[19] However, there is very minimal literature for use of this ubiquitous substance in implant dentistry. The authors here are trying a novel pilot study, of testing the effects of local application of this ayurvedic herbal remedy as an osseoinductive material to biofunctionalize the implant surface.

The aim of this study was to evaluate the osseointegrative potential of implants coated with or without *C. quadrangularis* hydrogel through evaluation of BIC and BAFO of implant titanium to the bone in the rabbit model.

Materials and Methods

The following experiment was done in the biotechnological laboratory and animal house facilities associated with the RajaRajeswari Group of Institutions, Bengaluru, Karnataka, India. The procedures were sequentially followed as elaborated below.

Preparation of *C. quadrangularis* Hydrogel

C. quadrangularis Aqueous Extract

Dried stems of *C. quadrangularis* were obtained from Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru, Karnataka, India. The stems were then ground and pulverized into powder and kept at 45°C in a hot air oven to remove any moisture. Moreover, 10 g of samples were taken for the aqueous extraction using the Soxhlet extraction method with water as a solvent. Further, the extract was rotary evaporated to obtain a thick concentrate of about 2 gm. The hydrogel was prepared using PEG obtained from commercial HImedia, India in the ratio 2:8 w/w%; extract:PEG. The extract was sonicated at 30°C for 45 minutes for even mixing to form a viscous hydrogel. The freshly prepared hydrogel was stored in sterile vials at 5°C until further use.

Animal Model

Institutional animal ethical clearance was obtained for the study (RRMCH/IAEC/4718/01). Six healthy loped ear male New Zealand rabbits weighing 3–5 kg were used in the study. Rabbits were fed with standard pelleted food and water and kept in the animal facility center of the institution. All the rabbits were kept under the required humidity and temperature and were monitored in a standard laboratory environment with a 12-h light and dark cycle. The in-house veterinary surgeon regularly recorded and maintained the vital reading. Intramuscular injection of 0.15 mL/kg Xylazine hydrochloride (Xylaxin, Indian Immunologicals, Hyderabad, Telangana, India) and 0.35 mL/kg Ketamine hydrochloride (Ketalar, Pfizer, Mumbai, India) was given as general anesthesia.

All the implants placed in this study were divided into two main groups – group I (control) and group II (test). Each group was then further divided equally into two groups based on the site of placement as either femur or tibia. The detailed distribution of the implants is tabulated in Table 1. Four implants were placed in each rabbit. Two each in each hind leg, one each in the femur and tibia as shown in Figure 1. The distribution within the rabbit was such that each rabbit received two coated implants (test) one in the femur and tibia and two uncoated implants (control) one in the femur and tibia. Implants were placed in each rabbit using a randomization program for their distribution (randomizer.org). The surgical assignment was done by a person not involved in the study using sealed envelopes. The assignment was informed to the surgeon immediately before

Table 1: The implant specimens divided into groups

Description	*Femur*	*Tibia*	*Total*
Group I: Without hydrogel (control)	6 implants	6 implants	12
Group II: With hydrogel (test)	6 implants	6 implants	12
	12	12	24

Fig. 1: Clinical photograph showing the placement of the implants in the femur and tibia of the rabbit hind leg

implant placement. The histological analysis was done by a different person who was not involved in any other section of the study.

A total of 24 titanium external hexed machined implants were used in this study. A total of 12 implants were used as control (group I) and the other 12 as test implants dipped in the hydrogel (group II). The dimensions were 3.75 mm diameter and a height of 8.5 mm (P-I Branemark Philosophy, Brazil). The surfaces of the coated and uncoated titanium implants were analyzed by a scanning electron microscope (SEM) to observe the surface changes on the implant surface.

Implant Surgery

Hind legs were shaved and disinfected with 70% ethanol and 70% chlorhexidine before surgery. Careful opening of the periosteal flap was done, and a standardized drilling protocol was followed to prepare the implant sites. The implant was placed after a sequential osteotomy procedure was carried out as per the instruction of the implant company. The test implants were coated with the CQ hydrogel just before placing into the osteotomy site (Fig. 2). The osteotomy site was sutured, and post-surgical antibiotics (Difidox, fusion healthcare Pvt. Ltd., India) and analgesics (INAC, Cadila Healthcare Ltd., India) were administered. The rabbits were constantly monitored until they became normal again. The rabbits were kept in the cage for 4 weeks after which they were euthanized with a high dose of sodium pentobarbitone (60 mL/kg) (Rematal, Taj Pharma Ltd., India). The implants along with the bone surrounding it were retrieved *en bloc* for further investigation. The following experiment was carried out in accordance with the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines.

Tissue Processing

The implant and the surrounding bone tissue obtained were stored in 10% neutral buffered formalin. The specimens were dehydrated in increasing ethanol concentrations from 70–100%. They were then cleared using an acetone alcohol mixture and embedded in methylmethacrylate (MMA) (Sigma–Aldrich, Burlington, MA, USA) after polymerization in MMA, thin sections of 70–100 microns were cut from the polymethyl metacrylate (PMMA) block using a precision microtome (Accutome-100, Struers, Denmark). These sections were mounted on a glass slide, ground further and the surface was polished with a speed grinder polisher (EcoMet 3000, Buehler, Germany). These sections were stained with hot Stevenson's blue and van Gieson's Picrofuchsin. Stained sections were evaluated in a trinocular transmitted light microscope (Nikon Eclipse Ni) and photomicrographs were captured using the camera (Nikon DS-Ri1) attached to the microscope. The BIC and BAFO were measured with the help of "Image J" software.

Fig. 2: Dip coating the implant with hydrogel. Observe the glistening wet surface of the implant

Histomorphometry

While calculating the BIC, the first three threads of the implant which are in contact with the bone were taken into consideration. The implant surface was mapped with the markers in the image J software and measured. The areas where these surfaces were contacted by bone were similarly marked and measured. The BIC was thus obtained.

Bone area fraction occupancy—the first three threads of the implant which were in contact with the bone—was analyzed. The area in between the consecutive threads within the diameter of the implant was measured. The area of bone fills in the above-marked area was considered the percentage of bone fraction occupancy.

The results obtained were then tabulated and later statistically analyzed using paired *t*-test.

Results

Scanning Electron Microscope Interpretation

Scanning electron microscope analysis of the surfaces of coated and uncoated implants was done. On observation, the roughened machined surface with irregular spines can be observed in the control implants (Fig. 3). The CQ hydrogel dip-coated test implants, however, show a relatively smoother surface with large spicules unevenly distributed, suggesting the conglomeration of the CQ hydrogel-forming an extra layer over the machined surface of the implant (Fig. 4).

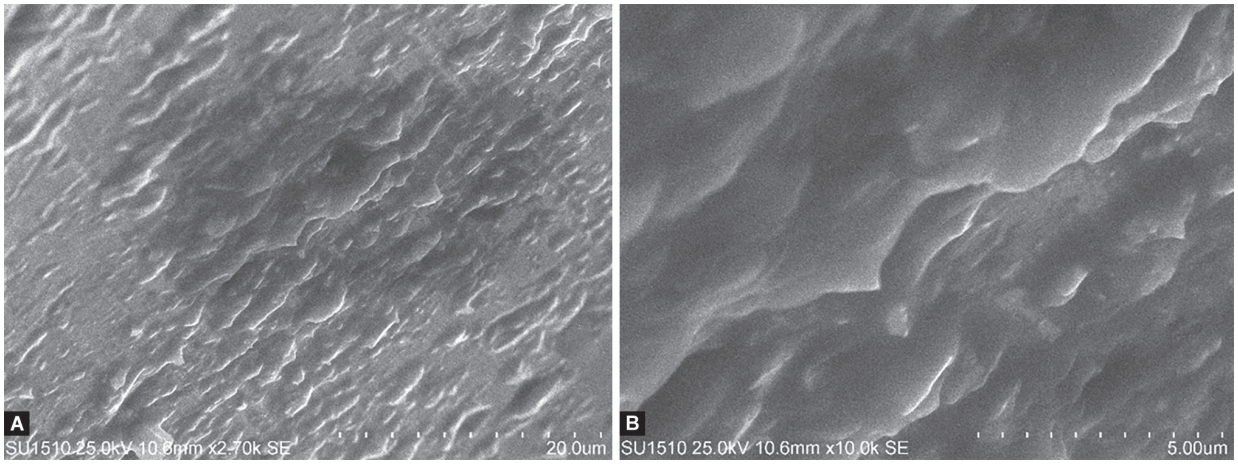
Histological Interpretation

There was a presence of the cortical bone in the crestal area of the implant suggesting that the cortical bone of the long bone was intact and cancellous bone was seen around the implant. Both the test and control implants showed new bone formation around the implant. There was no presence of inflammatory infiltrate around the implant. There was a marked increase in the areas of new bone formation in the femur as well as tibia in the test implants (Figs 5 and 6).

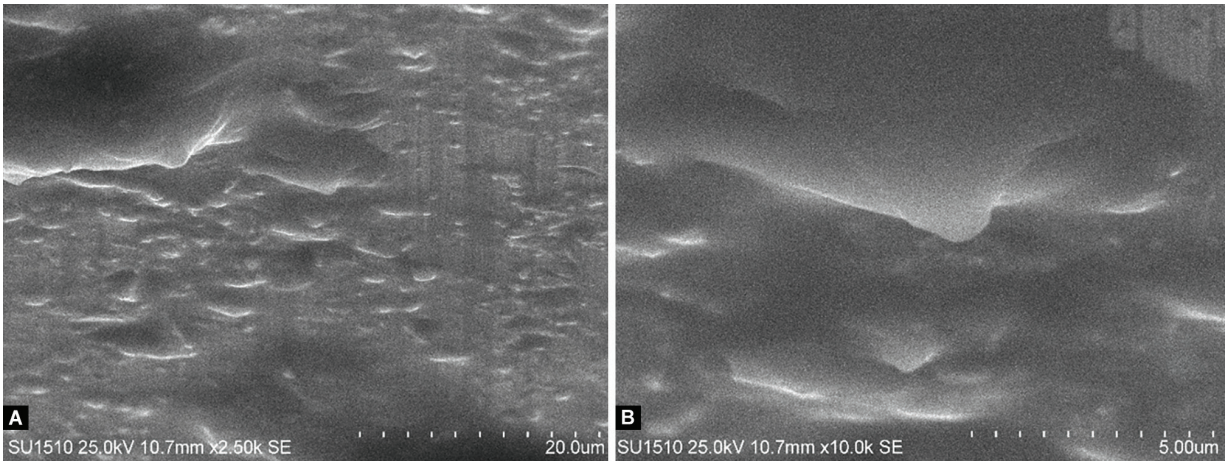
The BIC was calculated according to the method described in the methodology; the mean values obtained were tabulated (Table 2); the mean and the standard deviation (SD) values for the test femur were 60.29 and 14.74, respectively, which was greater than the mean BIC of control group 48.08 and 18.62. Similarly, there was a slight difference in the mean values in the tibia test group and tibia control group 56.0 and 52.06, respectively.

The paired *t*-test was the statistical analysis that was performed to check for significance. The resultant $p > 0.05$ indicates that the BIC values of the femur test group were not significantly different from the control group.

Bone area fraction occupancy values were calculated using the "Image J" software and the results obtained were tabulated (Table 3). The mean and SD values for the test femur were 64.45 and 14.30, respectively, and that of the control femur were 39.66 and 14.64.) paired *t*-test was performed to analyze the results for significance between the two groups with gel and without gel. The



Figs 3A and B: The SEM photographs of the non-coated control implants. (A) The outer surface of the machined implants showing discrete patches of roughness of the surface. (2,700×); (B) The same outer surface of the machined implants further magnified. *Note:* On the rough surface, many uniform spike-like projections and depressions can be clearly seen (10,000×)



Figs 4A and B: The SEM photographs of the test implant surface coated with CQ hydrogel. (A) The outer surface of the machined implants with the coated *C. quadrangularis* hydrogel. *Note:* The discrete viscous drop-like aggregations of hydrogel can be clearly seen (2,500×); (B) The same outer surface of the machined implants with the coated *C. quadrangularis* hydrogel further magnified shows the irregular roughness of the implant (10,000×)

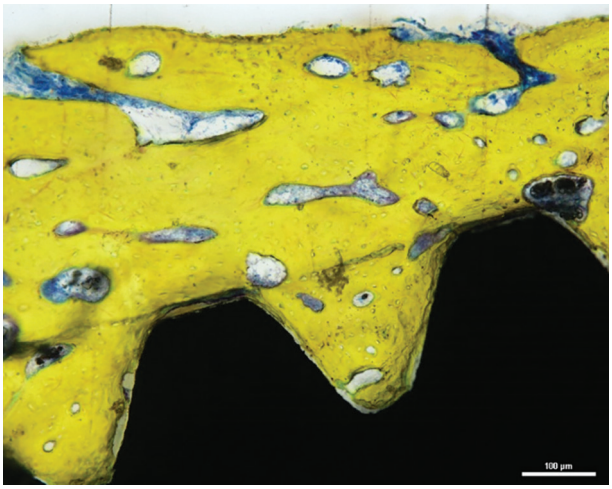


Fig. 5: The histomorphometrical image of the bone implant interface in the rabbit femur (stained with hot Stevenson's blue and von Gieson's Picrofuschin, 20×)

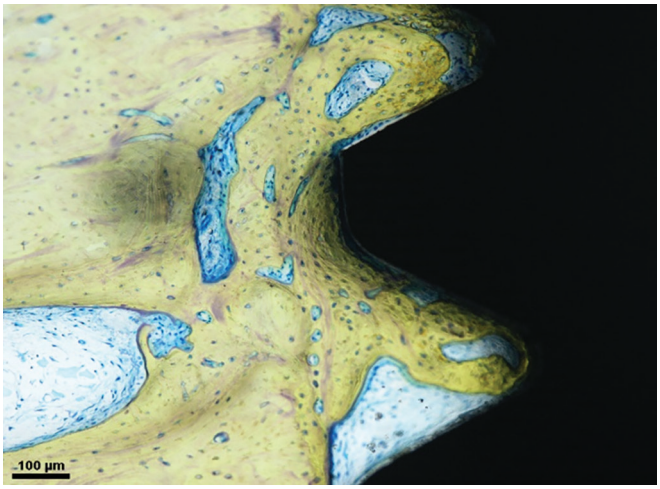


Fig. 6: The histomorphometrical image of Rabbit test femur showing the signs of islands of new bone formation (stained with hot Stevenson's blue and von Gieson's Picrofuschin, 20×)

Table 2: For BIC values for the test and control implants

Specimen	*Description of the group*	*Mean*	*SD*	*p-value*
Femur	Group I: Femur control (without gel)	48.08	18.62	0.131 (p >0.05)
	Group II: Femur test (with gel)	60.29	14.74	
Tibia	Group I: Tibia control (without gel)	52.06	17.44	0.630 (p >0.05)
	Group II: Tibia test (with gel)	56.0	17.27	

Table 3: The BAFO values for the test and control implants

Specimen	*Description of the group*	*Mean*	*SD*	*p-value*
Femur	Group I: Femur control (without gel)	39.66	14.64	0.003* (p <0.05)
	Group II: Femur test (with gel)	64.45	14.30	
Tibia	Group I: Tibia control (without gel)	43.60	10.87	0.37 (p >0.05)
	Group II: Tibia test (with gel)	49.42	15.45	

*Indicates significant difference between the two groups

difference between the femur test group and the femur control group was found to be significant (p <0.05). This indicates that the hydrogel-coated implants offered better bone growth and osseointegration than the control implants.

However, the test tibia did not have significantly different values than the control tibia group (p >0.05). The mean and SD values of the test tibia were 49.42 and 15.45, respectively, and that of the control implants were 43.60 and 10.87, respectively.

The results suggest that the test implants were significantly better in osseointegration in the femur rather than in the tibia. Although the mean values suggest that the osseointegration is not far behind in the tibia test implants. According to the results of the present study, we can safely conclude that the test implants with the CQ hydrogel had better BIC and BAFO values than the control implants. Since these values indicate for improved osseointegration, the *C quadrangularis* hydrogel promotes early and faster osseointegration.

Discussion

C. quadrangualris is a potent anti-osteoporotic,[20] anti-arthritic[21] therapeutic, and culinary[22] agent as the various research studies suggest. Several cell line and animal studies have proved this effect. It is mainly attributed to its potent bioactivity. This activity is due to the presence of phytochemicals that activate in different pathway mechanisms to enhance osteogenecity. The *C. quadrangularis* extract contains minerals,[23] and calcium oxalates,[24] which help in faster bone regeneration. The stem contains flavonoids and other substances also which enhance the biological properties of the CQ.

The parenteral/oral route administration has been tried and tested and it has resulted in faster healing time for jaw fractures. One recent study by Altaweel et al.[25] also uses this ability of *C. quadrangularis* to enhance osseointegration of implants placed in alveolar ridge distraction in atrophic mandibles. The authors observed that the distraction of the ridge was faster and healed better with the *C. quadrangularis* supplementation.

However, no studies have reported direct blood contact or local tissue application of the *C. quadrangularis* to the surgical site. This study here attempts to validate this beneficial effect on the direct contact application. The osseous cell line studies do indicate that the bioactivity of the *C. quadrangularis* is very good, the authors have also conducted a similar study and have got the same results of enhanced bioactivity *in vitro*.

The result obtained in this study suggest that the presence of *C. quadrangularis* hydrogel does provide better BIC. The test implants with the *C. quadrangularis* hydrogel did not demonstrate a significant increase in the BIC area. However, the BAFO values in the femur differed significantly suggestive that the bone growth was better than the control implants The results show that the test implants were significantly better in the femur suggesting that the use of this CQ hydrogel could speed up osseointegration in challenging areas of the jaw and give predictable osseointegration in corticocancellous bone. The use of the gel in deficient D1 and D2 bone or loose trabecular bone such as the posterior maxilla and the sinus areas still needs to be further validated.

C. quadrangularis contains minerals and calcium in abundance which may stimulate the osteoprogenitor cells to deposit bone over the titanium surfaces. The PEG hydrogel acts as a medium or scaffold for interaction of the blood and the *C. quadrangularis* and thus enhances the BIC and BAFO of the test implants and thus faster osseointegration.

The results obtained in this study are very similar to the results obtained in a study by Pachimalla et al.[26] who used a similar hydrophilic PEG gel incorporated with commonly used herbal material, acemannan, and moringa oleifera. The methodology was similar to the gel-coated test implants inserted in rabbit bone and tested later (after 4 weeks) for BIC values and bone volume (BV) values to determine osseointegration. The authors had found no difference between the test and control implants but there was an increase in the mean BIC in the test implants. The hydrophilic gel used in the study was made from acemannan and moringa oleifera which are active ingredients extracted from the aloe vera, a commonly found herbal remedy. The results encourage to investigate other Ayurvedic herbal plant extracts of common day-to-day use which could have osteogenic potential.

Campbell and Duncan[27] and Duncan[28] et al. studied a similar study on the effect of keratin hydrogel coated implants in animal models by observing an increase in the stability values and increased BIC value sin histomorphometry. They thus concluded that the keratin hydrogel induced better osseointegration. The authors hypothesized that the keratin in a hair follicle had regenerative potential because it did have the growth factor, cytokines and signaling processes all of which could induce osseous regeneration as well if applied to the bone. The authors found similar results in the sheep model as well.

Dip coating is one of the many methods wherein a substrate is coated on the implant surface. Many researchers have tried dip coating titanium surfaces with different antibiotic gentamicin gels[29] and titanium salts in polymers[30] or silica PEG hydrogel hybrids[31] all with an intention to enhance the bioactivity of the titanium surface. Our study here employs the same dip coating method to increase the bioactivity of implants.

Pan et al.[8] have used the scaffolding effect of BMP-2 hydrogel in an animal model and found that the BMP-2 induced bone regeneration is faster than the normal implants. the resultant

histomorphometry proves that the effect of the BMP-2 as an osteogenic agent is beneficial. The results of these studies and similar studies suggest the use of a combination of the osseoinductive material such as recombinant human bone morphogenic protein (rhBMP) and bTCP which is delivered on the surface of the titanium implants to enhance the osseointegration. The hydrogels act as a scaffold for the easy ingress of the progenitor cells into the contact the titanium. The authors here have tried to simulate the same effect with the *C. quadrangularis* extract replacing the BMP in the hydrogel.

The limitations of this study could be that the animal model used here was of a limited number which cannot be extrapolated to a larger human population. The results obtained in this study need to be replicated in another higher animal model as well to have a clear understanding of the effects. Although the architecture of the rabbit bone most closely mimics the human bone, the human bone is denser and made of a complex osseous structure. Similar studies need to be carried out on a larger scale and human trials need to be carried out to test the efficacy of the CQ hydrogel in local applications.

Conclusion

Based on the limitations of this study we can conclude that the hydrogel of the *C. quadrangularis* enhanced the bone growth around the implants in the experimental rabbit model. The findings in this study are an initial validation for herbal hydrogel-enhanced osseointegration in the bone tissue. It was more pronounced in femur so we can safely conclude that the hydrogel is beneficial in strong bone foundations. Further studies need to be done to validate the beneficial effect of these ayurvedic medications to be used as osseoinductive materials used in the faster osseointegration of titanium to the bone.

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