Research Article

Osseointegration effects of whey protein (histological and histomorphological observations): An experimental study on rabbits

Nawar Bahjet Kamil [1*], **Nada M.H. AL-Ghaban** [1], **Amaar Aamery** [2]

1 Department of Oral Diagnosis, College of Dentistry, University of Baghdad, Baghdad, Iraq.
2 Senior Clinical Fellow. University Hospital Coventry & Warwickshire NHS Trust, England.
*Corresponding author: nawar.bahjat@codental.uobaghdad.edu.iq,

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Abstract: Background**:** Whey protein is the green-yellow colored, liquid portion of the milk, and it is also called the cheese serum, it is obtained after the separation of curd, during the coagulation of the milk. It contains a considerable amount of α-helix pattern with an evenly distributed hydrophobic and hydrophilic as well as basic and acidic amino acids along with their polypeptide chain. The major whey protein constituents include β-lactoglobulin (β-LG),α-lactalbumin (α-LA), immunoglobulins (IG), bovine serum albumin (BSA), bovine lactoperoxidase (LP), bovine lactoferrin (BLF) and minor amounts of a glycol macro peptide (GMP). Osseointegration can be defined as a process that is immune driven which leads to the formation of the new bone surrounding the surface of the implant rather than a pure response of the bone. Titanium can activate a balance recognized to be tolerogenic with a peri-implant tissue leading to a "foreign body equilibrium (FBE)" response. Materials and methods: Twelve adult male white New Zealand healthy rabbits were used in this study, the animals were divided into two groups according to the time of scarification as follows; 2 and 6 weeks after the implantation (6 rabbits will be sacrificed for each group). Results: Statistical analysis showed that there is a highly significant difference in all parameters between the experimental group and control group at 2 weeks and 6 weeks periods. Histological results at 2 weeks period showed thread formation in whey protein and control group, distribution of osteocyte cells and osteoblast was higher in whey protein, and the bone trabecular area was also larger in whey protein groups but at 6 weeks showed mature bone in whey protein groups while in control group still woven bone. Conclusions: Whey protein is an effective in osseointegration because it enhances bone formation.

Keyword: whey protein, osseointegration, histologically and histomorphological.

Introduction

Osseointegration can be defined as a process that is immune driven which leads to the formation of new bone surrounding the surface of the implant rather than a pure response of the bone [(1)]. Titanium can activate a balance recognized to be tolerogenic with a peri-implant tissue leading to a "foreign body equilibrium (FBE)" response [(2)].Following this, an immune response will regulate wound/tissue healing and enhance the regeneration mechanism [(3)]. Mechanisms of osseointegration include the Inflammatory phase: platelets are exposed to the implant and then released histamine and serotonin causing platelet aggregation and thrombosis these are called vascular events and the clotting process begins when the blood contacts the foreign materials or proteins[(4)]. Proliferative phase: In this phase, vascular growth comes from the surrounding tissue as a process called neovascularization. Mesenchyme cells are triggered to differentiate into (fibroblast, osteoblast, and chondroblast) [(5)].Maturation phase: This phase occurs with the ossification of the fibrocartilaginous callus and continuous deposition of woven bone in the peri-implant space by

differentiation of mesenchyme cells in the granulation tissue) [6]. Whey protein is the green-yellow colored, liquid portion of the milk, and it is also called the cheese serum, and It is obtained after the separation of curd, during the coagulation of the milk [7]. It contains a considerable amount of α-helix pattern with an evenly distributed hydrophobic and hydrophilic as well as basic and acidic amino acid along with their polypeptide chain [8]. The major whey proteins constituents include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins (IG), bovine serum albumin (BSA), bovine lactoperoxidase (LP), bovine lactoferrin (BLF) and minor amounts of a glycomacropeptide (GMP). The whey protein is of benefit as it provides immunity support, boosts metabolism, increases muscle mass and helps to improve overall health. The whey protein promotes (Muscle synthesis, Muscle strength, Performance, and endurance, improves the immune system, and more favorable body recovery and composition [9].

Materials and methods

Twelve adult male white New Zealand healthy rabbits were used in this study, aged between 10-12 months, and their weight ranged between 2-2.5 kilograms. The rabbits were all housed with controlled temperature and ventilation conditions and were all given a standard diet (pellet and berseem) with tap water that was easily accessed. The rabbits were kept in a separate standard cage according to the decision of the approval committee of the College of Dentistry/ University of Baghdad (531). The animals were divided into two groups according to the time of scarification as follows; 2 and 6 weeks after the implantation (6 rabbits will be sacrificed for each group). First, General anesthesia was induced by intramuscular injection of ketamine hydrochloride with a dose of (1 ml/kg of body weight) plus xylazine [10]. Pure Titanium (cpTi) implants are placed on each rabbit's left and right side of the femur.

The uncoated one was placed on the right side and coated implants with whey protein were implanted in the left femur, By intermittent drilling and with continuous cooling by irrigation of normal saline, bone preparation was performed by a micro engine with a rotary speed of 2500 rpm, the openings were made by a round bur, the holes were about (1.8 mm in diameter) and were enlarged gradually with a spiral drill from 2-2.5mm till the final dimension of 3.0mm, then the operation sites were washed by normal saline for the removal of the debris from the sites of drilling. The sterilized implants were placed in the beds, the insertion of the uncoated one was directly done while the insertion of the coated implants was performed after the application of whey protein. Later, the implants were placed in the holes by a screwdriver designed specially to fit the screws slit and then threaded until the screw's threads were completely introduced into the bone tissue [11]. All the specimens were prepared for histological and histomorphological study, measuring the account of osteoblast, osteocyte, and area of bone marrow, trabecular, and new bone formation using image J [12].

Result

Statistical analysis showed that there is a highly significant difference in all parameters between the experimental group and the control group at 2 weeks period. Experimental groups recorded a higher mean value in osteoblast cells account and also recorded a higher mean value in osteocyte cells account. The bone formation area recorded higher mean values in the experimental groups than in the control groups. Bone trabecular area recorded a higher mean value in the experimental groups than the control one but bone marrow area recorded a higher mean value in the control groups than the experimental groups as shown in Table (1).

Table 1: The osteoblast and osteocyte cells account and new bone formation, bone marrow, and trabecular area at 2 weeks period.

2 week/ Osteoblast Cell	W	C	p-value1
Mean	43.25	21.17	0.01
Std. Error of Mean	1.52	1.02	
Median	43.80	20.35	
Std. Deviation	3.73	2.49	
2 week/ Osteocyte Cell			
Mean	17.98	8.98	0.01
Std. Error of Mean	0.59	0.83	
Median	17.75	9.01	
Std. Deviation	1.44	2.02	
2 week / New Bone Formation			
Mean	3.07	2.02	0.01
Std. Error of Mean	0.12	0.11	
Median	3.02	1.97	
Std. Deviation	0.30	0.26	
2 week /Trabecular area			
Mean	0.66	0.35	0.01
Std. Error of Mean	0.66	0.04	
Median	0.06	0.34	
Std. Deviation	0.65	0.10	
2 week / Bone marrow area			
Mean	0.39	0.54	0.01
Std. Error of Mean	0.39	0.04	
Median	0.06	0.58	
Std. Deviation	0.41	0.09	

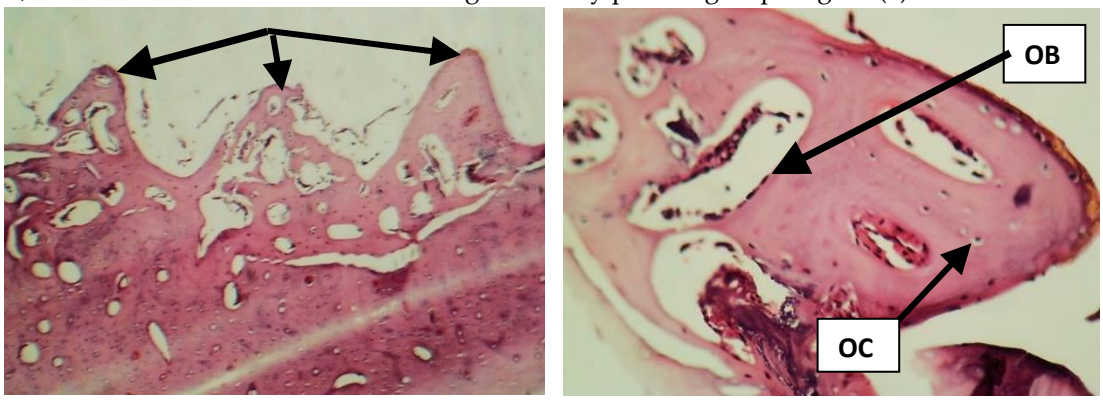
At 6 weeks period, there was a significant difference in all parameters between the whey protein groups and the control group. The control groups recorded a higher mean value in osteoblast cell account than the experimental groups but the experimental groups recorded a higher mean value in osteocyte cell account than the control groups. The experimental group recorded a higher mean value than the control groups in the bone formation area and also recorded a higher mean value in the trabecular area but in the bone marrow area the control group recorded a higher value as shown in table (2).

Our results showed that the osteoblast cells account was decreased in both groups with time but the osteocyte cells increased with time in both groups. The new bone formation area and the trabecular area increased with time but the bone marrow area decreased in both groups and there was a significant difference between the experimental groups and the control groups as shown in Table (1,2)

Table 2: The osteocyte and osteoblast cells account and new bone formation, bone marrow, and trabecular area at 6 weeks period

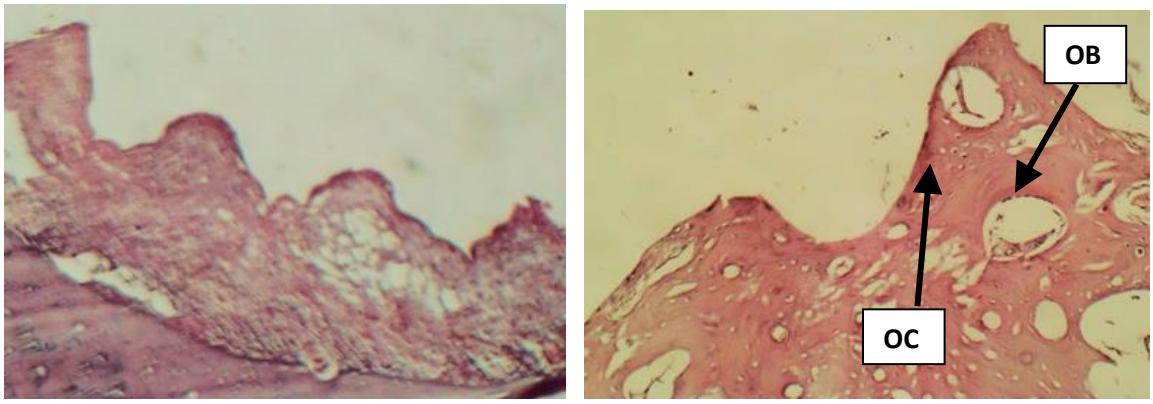
6 week/ Osteoblast Cell	W	C	p-value1
Mean	21.08	34.25	0.01
Std. Error of Mean	1.25	1.56	
Median	20.15	34.85	
Std. Deviation	3.05	3.82	
6 week/ Osteocyte Cell			
Mean	41.52	22.98	0.01
Std. Error of Mean	1.05	1.05	
Median	41.75	22.80	
Std. Deviation	2.56	2.58	
6 week / New Bone Formation			
Mean	5.05	3.51	0.01
Std. Error of Mean	0.13	0.17	
Median	5.04	3.35	
Std. Deviation	0.32	0.41	
6 week /Trabecular area			
Mean	0.87	0.46	0.01
Std. Error of Mean	0.03	0.04	
Median	0.86	0.48	
Std. Deviation	0.08	0.11	
6 week / Bone marrow area			
Mean	0.27	0.41	0.01
Std. Error of Mean	0.05	0.05	
Median	0.29	0.40	
Std. Deviation	0.13	0.11	

Histological result**: A** 2weeks period showed thread formation with fibrous tissue in whey protein and control group with osteoprogenitor cells, distribution of osteocyte cells and osteoblast was higher in whey protein, the bone trabecular area was also larger in whey protein groups Figure (1).



(A) **(B)**

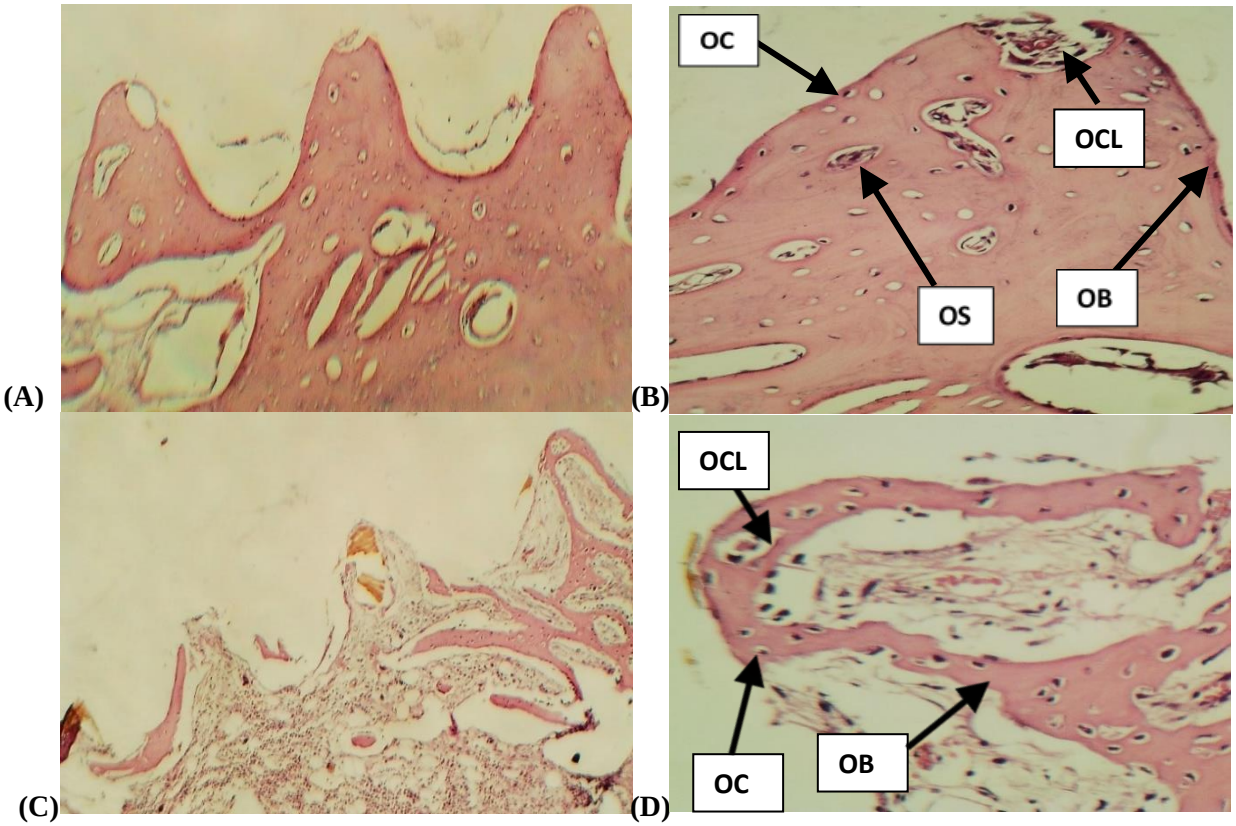
Figure 1:A: Thread formation (black arrow) in whey protein X(10) **B:** osteocyte (OC), osteoblast (OB) in whey protein X (40)



(C) **(D)**

Figure 1: C: Thread formation in control group X(4) **D:** osteocyte (OC), osteoblast(OB) in control group X(20) in control group

Histological features at 6 weeks period showed mature bone with lamellar bone formation in whey protein groups but still immature woven bone in the control group. The account of osteocytes in both groups was increased because of the embedding of osteoblast in osteoid and the formation of osteocytes. Osteoclast cells were observed in both groups with osteon formation and an increase in the thickness of the trabecular bone as shown in Figure (2).



(A) **(B)** **(C)** **(D)**

Figure 2: A: mature bone in whey protein groups X(10) **B:** osteoclast(OCL), osteoblast(OB), osteocytes (OC), osteon (OS) X(40) in whey protein, **C:** woven bone with trabecula in control group X(10), **D:** osteoclast(OCL), osteoblast(OB), osteocyte(OC), X(40) in control group.

Discussion:

Osseointegration is defined as a process that is driven by the immune response, which can lead to the new bone formation that surrounds the surface of the implants rather than as a pure response of the bone. Osseointegration is a dynamic process that can result from complex reactions and interactions that allow the integration of the bone and the oral mucosa [1].

Titanium implant was used in the current study because of their biological and chemical properties, good bioactivity, and ability to osseointegrate [13].

The Whey protein consists of lactalbumin, lactoglobulin, light and heavy-chain immunoglobulins, lactoferrin, bovine serum albumin, glycomacropeptide, and lactoperoxidase. So, it has antimicrobial, antibacterial, anti-carcinogenic, and immune modulation activity. Also, it contains an essential amino acid and a high percentage of sulfur amino acids that has antioxidant property and for that reason, it had been chosen in our study [14,21].

At 2-week intervals, there was threads formation filled by soft tissue and inflammatory cells with progenitor cells which are the precursor for the osteoblast cells in all groups with various rates of bone formation which was more with whey protein due to the increased production of T cells which reduces the inflammatory cells, prevent bacterial infection and allergy which help osteoprogenitor cells to differentiate into osteoblast cells and then bone formation [22].

Osteoblasts were observed on the surface of the thread and lining the bone trabecula and recorded a higher value in the experimental groups and a highly significant difference between them this agrees with Mohammad and Al-Ghaban [11] and indicated the activity of whey protein in the formation of woven bone. That is related to osteogenic differentiation and enhancement of cell proliferation [15]. Osteocytes recorded a higher value in the experimental groups than the control groups and this was because of woven bone formation and this agrees with Jawad and Al-Hijazi [16].

Osteocyte cells recorded a highly significant difference in the experimental groups than the control one and were indicated for impingement of osteoblast cells by newly formed bone and converted to osteocyte cells which are important in the regulation of bone formation and resorption.

The current study revealed that the trabecular bone was thicker and more with whey protein groups than the control groups. Whey protein can influence the immune cells, antibodies, secretion of cytokines, granulocytes, phagocyte activity, and the activity of the natural killer cell [23] This was capable of accelerating trabecular bone formation. This study illustrated that bone marrow area in whey protein groups was smaller than the control groups which was indicated by more bone formation in the experimental groups due to its anti-oxidant effects which increase the antioxidant effect of peroxidase, catalase, glutathione, and superoxide dismutase [24].

At 6 weeks, osteoblast cells account was higher in the control group than in the experimental groups which was indicated by the impeded osteoblasts in the osteoid and then converted to osteocyte cells and with a high rate of bone formation in the experimental groups. This was related to the anti-bacterial and antimicrobial effects[18] and this agrees with Mahmood and Al-Ameer [17]. As a result, osteocyte cells account increase in whey protein groups more than the control groups and this agrees with Mohammad and Al-Ghaban [11] who cleared that osteocyte cells increased with time while osteoblast cells decreased with time.

New bone formation area was recorded to be larger in whey protein groups due to lamellar bone formation which was still immature woven bone in the control groups and that may be because of its antioxidant,

antimicrobial effect, and immune enhancement properties related to lactoferrin, immunoglobulin, serum albumin, glycomacropeptide and lactoglobulin content [19].

The trabecular bone area was thicker in whey protein groups and this was related to the mature bone formation as compared to the control groups which was still immature bone at this time. Bone marrow area recorded a lower value in whey protein groups than in control groups due to the deposition of bone and formation of osteon related to the anti-oxidant and osteogenic activity of the whey protein as compared to control groups [24,15] and this agrees with Al-Azzawi And Al-Ghaban [20].

From this study we noted that with time osteoblast cells account decreased and osteoblast cells account increased, new bone formation area and trabecular bone was increased but bone marrow area was decreased because of the Osseointegration process and this agrees with Mohammad and Al-Ghaban; Mahmood and Al-Ameer [17,11] and there was a significant difference between the experimental groups and the control groups in all parameter because of the antibacterial, anti-inflammatory, antioxidant and immune modulation activity of whey protein [14,24].

Conclusion:

Whey protein is an osteoconductive material for the enhancement of osseointegration by increasing osteoblast differentiation and decreasing the osteoclast genesis, and this study showed early deposition of osteoid tissue in whey protein-coated implants. So, whey protein is effective in osseointegration and it is easy to obtain and use because it is inexpensive.

Conflict of interest: None

Author contributions

NN; study conception and design, data collection, Methodology. NNA; statistical analysis and interpretation of results. NN; original draft manuscript preparation. NNA; Writing - review & editing. Supervision. All authors reviewed the results and approved the final version of the manuscript to be published.

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Informed consent

The study was approved by the College of Dentistry/University of Baghdad's local ethics commission (project No. 531722, Ref. number: 531).

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العنوان: تأثيرات الاندماج العظمي لبروتين مصل اللبن (نسيجيا) دراسة تجريبية في الارانب

الباحثون: نوار بهجة كامل , ندى محمد حسن الغبان , عمار العامري

المستخلص: بروتين مصل اللبن هو الجزء السائل من الحليب ويسمى أيضا مصل الجبن يحتوي كمية كبيرة من نمط الحلزون بالإضافة الى الاحماض الامينية الأساسية والحمضية مع سلسلة البولي ببتايد الخاصة بهم. يمكن تعريف الاندماج العظمي بأنه عملية مناعية والتي تؤدي الى تكوين عظم جديد يحيط سطح الغرسة يمكن للتيتانيوم ان يعمل توازن حيث انه معروف للتحمل مع نسيج شبه مزروع يؤدي الى الاستجابه للتوازن.

في هذه التجربة تم استخدام اثني عشر من الارانب البيضاء النيوزيلندية السليمة وتم تقسيم الحيوانات الى مجموعتين اسبوعان وستة أسابيع كل فترة يتم اخذ العينات من ستة ارانب . اظهر التحليل الاحصائي وجود فرق معنوي كبير في جميع المتغيرات بين مجموعة التجربة ومجموعة السيطرة في الفترات المقررة . أظهرت النتائج النسيجية بعد أسبوعان بداية تكوين العظم في كلا المجموعتين وكان توزيع خلايا العظم اعلى في مجموعة مصل اللبن من محموعة السيطرة كما كانت منطقة تكون العظم الجديد اكبر في بروتين مصل اللبن اما بعد ستة أسابيع فقد أوضحت الدراسة تكون عظم ناضج في مجموعة بروتين مصل اللبن ولكن مايزال العظم غير ناضج في مجموعة السيطرة . نستنتج من هذه الدراسة بأن بروتين مصل اللبن فعال بتسريع الاندماج العظمي.