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Autogenous tooth graft with platelet rich fibrin versus autogenous tooth graft only around immediate dental implant

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***Abstract*---**Objective: This trial was conducted to compare between autogenous fresh tooth graft with/without platelet rich fibrin around immediate dental implant. Patients and methods: This controlled randomized clinical trial was carried out on 12 patients over the age of 18 who required dental extractions of single- or multi-rooted teeth, who were also delayed candidates for osseointegrated implants. They were equally divided into two groups; group (1): Autogenous fresh tooth graft around immediate dental implant placement with platelet rich fibrin and group (2): Autogenous fresh tooth graft around immediate dental implant placement without platelet rich fibrin. Results: The mean value of horizontal bone loss in group (1) and

group (2) at 6 months 0.077 (0.065 -0.130) and 0.595 (0.450 - 0.690) respectively with statistical significant differences between two groups. While vertical bone loss in group(1) and group(2) at 6 months (0.510(0.480 -0.530) and (1.490(1.400 - 1.640) respectively with statistical significant differences between two groups. The mean value of implant stability quotient for group (2) at 3 months was 70.50 ± 3.56 ISQ, and increased to 77.67 ± 5.61 ISQ at 6 months, which was statistically significant. While in group(1) at 3 months was 82.83±5.53 ISQ and increase to 90.67±1.21 ISQ at 6 months, which was statistically significant. There was a statistically significant difference between the two groups. Conclusion: This procedure is beneficial for preserving alveolar bone and getting important, high-quality bone structure. Despite the more sophisticated equipment, the sticky tooth approach appears to be rather effective.

***Keywords*---**autogenous fresh tooth graft, platelet rich fibrin mixture, immediate dental implant placement.

Introduction

Because loss of bone height complicates oral rehabilitation following tooth extraction, bone height falls gradually by 25% throughout the first year following tooth extraction, with a total of 4 mm of loss in height thoughout this first year post-extraction. Significant changes in vascularization occur as a consequence of bone resorption, along with intrabony vascularization giving way to centripetal periosteal vascularization. These dimensional alterations in the alveolar process can render the insertion of an implant in a three-dimensional position problematic [1, 2].

To mitigate the unfavorable repercussions of tooth extraction, a variety of therapy options including immediate implants and non-resorbable xenograft biomaterial were applied [3]. Following tooth extraction, immediate implant implantation is a well-recognized and effective therapeutic option. Therefore a risk of losing vestibular bone height and soft tissue which is unacceptable from rehabitilation of view [4].

Accordingly several studies evaluated different types of bone graft to overcome the bone resorption for labial/buccal or lingual/palatal aspects, There are; 1- Autograft, 2- Allograft, 3- Xenograft, 4- Alloplastic graft, 5- Growth factors [5]. Autogenous grafts are always the gold standard and the benchmark of all graft types because of Its osteoinductive, osteoconductive, and osteogenic. However, secondary surgical sites, pain, high rates of donor site morbidity and insufficient graft material are the main disadvantages of autogenous graft [6, 7].

Therfore, there are several trials to overcome the bone resorption. Demineralized autologous dental dentin has been offered as a novel option to autogenous bone grafts due to its osteoinductivity in the same context. After decalcification and sanitation, a patient's removed teeth can be utilized as noble bone transplant material. which has been commonly utilized to augment the ridge and sinuses.

Dentin and alveolar bone are identical both chemically and histologically, and have the same embryologic origin. Thus, dentin may be employed as a temporary graft material that is eventually replaced by bone [8-11].

Several studies have found effectiveness with implants and fresh tooth grafts when PRF is used as a significant supply of numerous growth factors to accelerate bone development in the graft [12]. Choukroun et al. introduced platelet-rich fibrin in 2006 in France. It was deemed a second-generation platelet concentrate since it is made without anticoagulants or gelifying chemicals. PRF is totally autogenous in nature, simple to manufacture, and very affordable due to the fibrin's unique three-dimensional geometry. Additionally, PRF demonstrated elevated levels of pro-inflammatory and pro-healing cytokines.

Following activation of platelets trapped inside the fibrin matrix, growth factors are released. This stimulates the mitogenic response in the bone periosteum throughout the healing of normal wound. Since the last two decades, a deeper knowledge of the physiological features of platelets in wound healing has resulted in a rise in their therapeutic uses in a variety of forms with different results. PRF marks a watershed moment in the advancement of the platelet gel therapeutic approach [14].

Accordingly, this study was be a trial to evaluate the effect of autogenous fresh tooth graft with or without PRF on osteointegration of immediate dental implant. The aim of the present trial was to compare between autogenous fresh tooth graft with/without platelet rich fibrin on osseodensification of immediate dental implant.

Patients and Methods

Twelve adult patients of both sexes were included in this Randomized Controlled Clinical Trial. Each patient's severely destructed teeth need prompt extraction and implant insertion. All patients signed informed written consent and agreed to attend scheduled follow-up appointments. The patients were recruited from the Out Patient Clinic of the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Al-Azhar University - Assiut.
Group(1): Autogenous fresh tooth graft around immediate dental implant placement with PRF.
Group(2): Autogenous fresh tooth graft around immediate dental implant placement without PRF.

The study included healthy adult patients without systemic complications, patients who required tooth extraction due to root caries, periodontal disease or fractures but were also candidates for immediate replacement with an osseointegrated implant, good dental hygiene, non smokers or patients who smoked less than 10 cig/day. While the following criteria were used to exclude patients: periodontal or periapical infection that is active and affecting the teeth to be removed, heavy smokers, patients undergoing radiotherapy or chemotherapy, autoimmune disease, patients with uncontrolled systemic diseases like uncontrolled diabetes mellitus as well as subjects having parafunctional

behaviors such as clenching, bruxism, lip or fingernail biting and excessive gum chewing.

The Implant system:

The implant (Two-stage screw NucleOSS™ T6 Implant System, Turkiye, Izmir, Al-Masa Dental Store from Egypt) was put on a color-coded fixure mount, either as a transfer or as a short straight abutment.

Preoperative phase

All patients received a clinical examination pre-operatively: their data were obtained, including their name, age, and gender as well as their dental and medical history. The oral mucosa of the edentulous region was inspected and palpated. Additionally, all patients had standardized periapical radiography to rule out any periapical disease as well as a pre-operative panoramic radiographic assessment to determine the appropriate implants' size to be implanted using Cone beam computed tomography (CBCT).

Operative phase

All patients were asked to rinse their mouths for two minutes immediately before operation with chlorhexidine mouth wash (Listermix plus, SIGMA Pharmaceutical Industries, Egypt). Local anesthetic, articaine HCL, and epinephrine 1:20.000 (Septodont, by Novocol Pharmaceutical of Canada, Inc.) were used to treat all patients.

In group (1)

A) Tooth extraction;

The extractions were conducted atraumatically with manual periotomes to prevent altering the alveolar ridge during the extraction. Following that, a thorough alveolar curettage was performed. When a removed tooth required root canal treatment, it was not utilized as donor material (Figure 1).

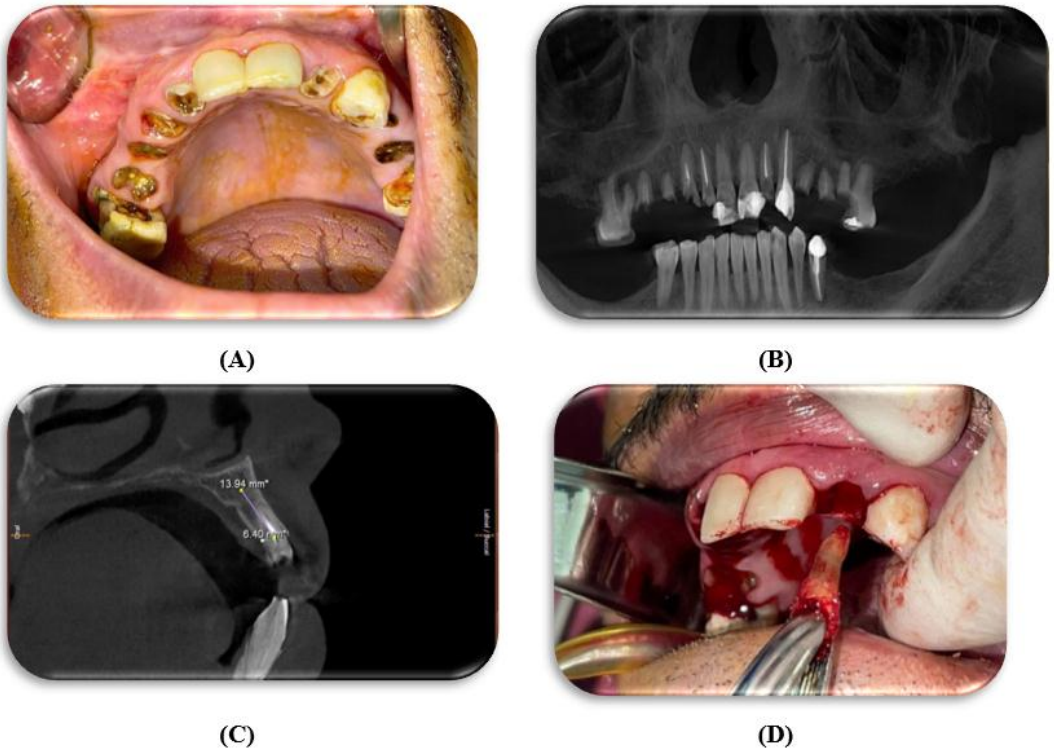


Figure 1: (A) Photograph showing preoperative case (B) Photograph showing preoperative Panoramic x-ray (C) Photograph showing preoperative sagittal CBCT (D) Photograph showing delivary of the the tooth after extraction

B) Tooth processing

Preparation and processing the tooth graft was done Immediately after extraction. Discolored dentin and carious lesions, as well as periodontal ligament (PDL) remnants and calculus were removed with a high speed tungsten carbide bur. In the case of multi-rooted teeth, the roots were divided (Figure 2). The cleaned teeth, roots and crowns included, were dried using an air syringe and ground with the newly designed 'Smart Dentin Grinder' (Kometabio device, United Kingdom, The Regen Store). Dentin particles with a diameter of 300-1200 µm were recovered. In a tiny sterile glass container, the particulate dentin from the drawer was submerged in basic alcohol for ten minutes. The particle was cleaned twice in sterile phosphate buffered saline after decanting the basic alcohol cleaner (PBS). The PBS was decanted leaving wet particulate dentin ready for grafting (Figure 3).

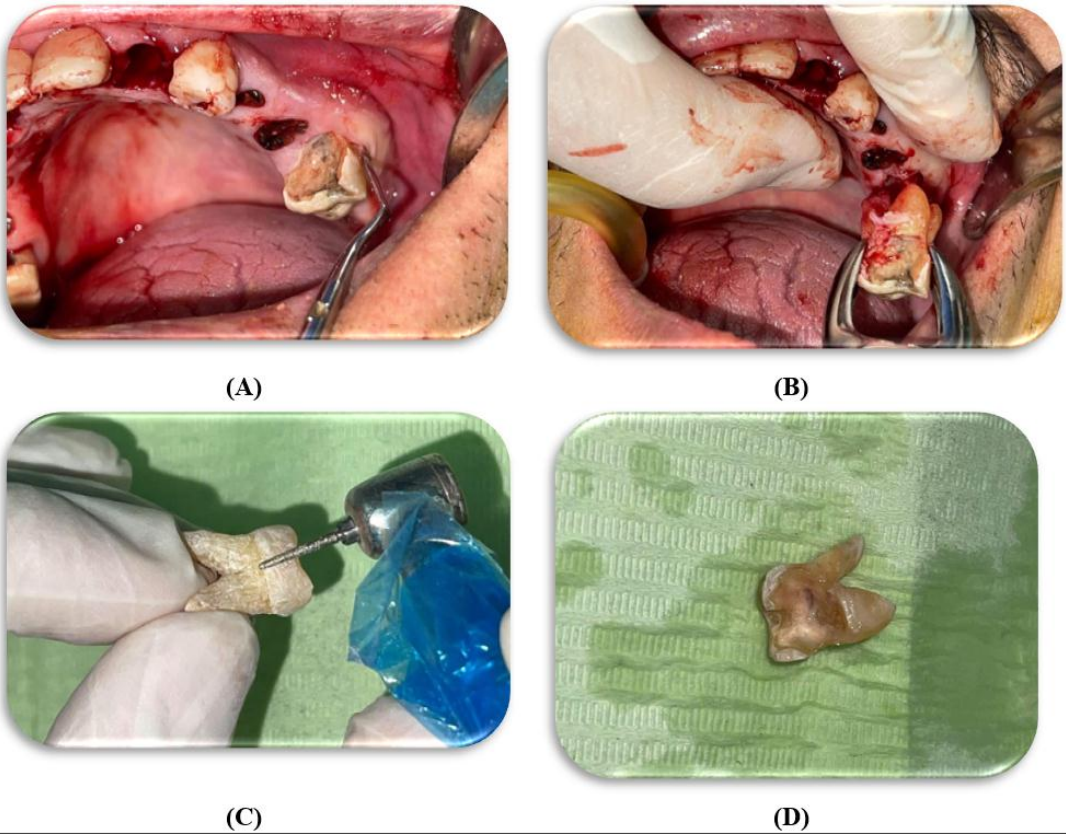


Figure 2: (A) Photograph showing application of periotome on the extracted fresh Autogenous tooth graft (B) Photograph showing delivary of the tooth after extraction (C) Photograph showing removing of the carious lesion by contra-angled high speed (D) Photograph showing extracted tooth after cleaning

Figure 3: (A)the Smart Dentin Grinder device (B) the autogenous fresh tooth graft in the cup of Smart Dentin Grinder (C) the grinded particles in the collecting chamber

C) Preparation of platelet rich fibrin;

Both of the 6 ml sterile vacutainer tubes were filled with about 5 ml of whole venous blood with no anticoagulant. For 10 minutes, the vacutainer tubes were centrifuged at 3000 revolutions per minute (rpm), following which it settled into three layers: the red bottom fraction containing red blood cells, the top straw colored cellular plasma, and the middle portion containing the fibrin clot. The upper straw colored layer was separated and the middle portion was collected at a depth of 2 mm below the bottom seperating line, which represents the PRF (Figure 4).

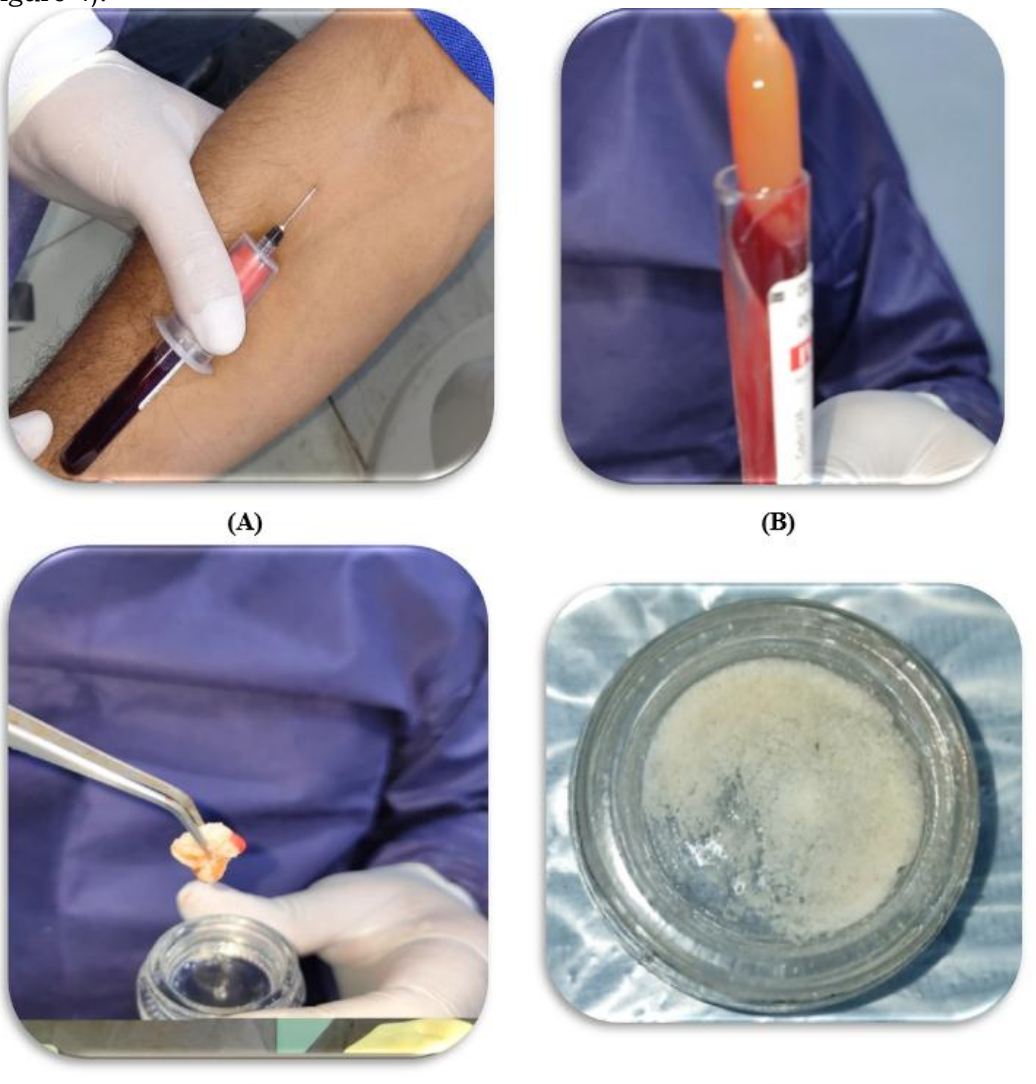


Figure 4: (A) collection of blood sample (B) PRF FIBRIN (C) sticky fresh autogenous tooth graft ready for augmentation around immediate dental implant (D) Fresh autogenous tooth graft without PRF

D) Implant Insertion

Drilling for implant placement with sequential drills, and the implant will be placed in fresh extracted socket using implant ratch. Ratchet was used to insert the implant and tight in its bed in a clockwise direction to the determine length. The tighting of implant using insertion torque of 50 Ncm (Figure 5). Smart peg was applied to implant to determine and read the primary stability with osstell machine.The cover screw was removed from the bottom of the implant vial by a hex tool and screwed into the implant body. The buccal and the palatal soft tissue were approximated and sutured by simple interrupted suture (Figure 6).

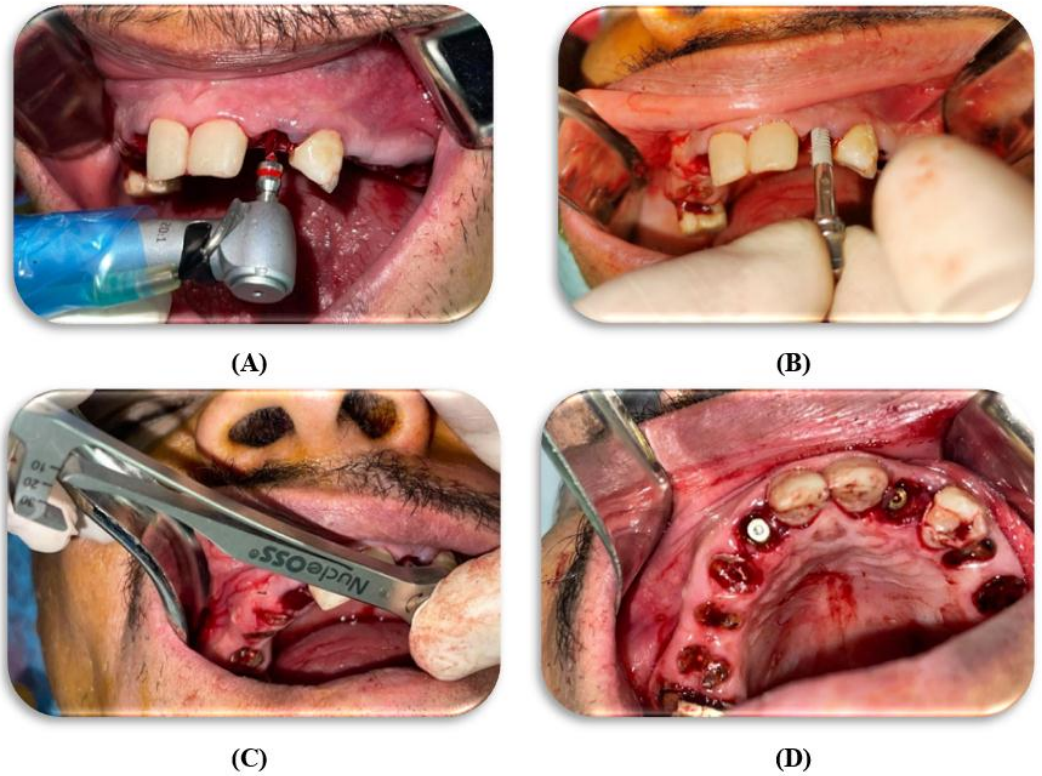


Figure 5: (A) preparation of the osteotomy site (B) screw of implant to prepared site (C) the implant was threaded in place using the ratchet in a clockwise direction (D) implant placed palatal to that site after ratching

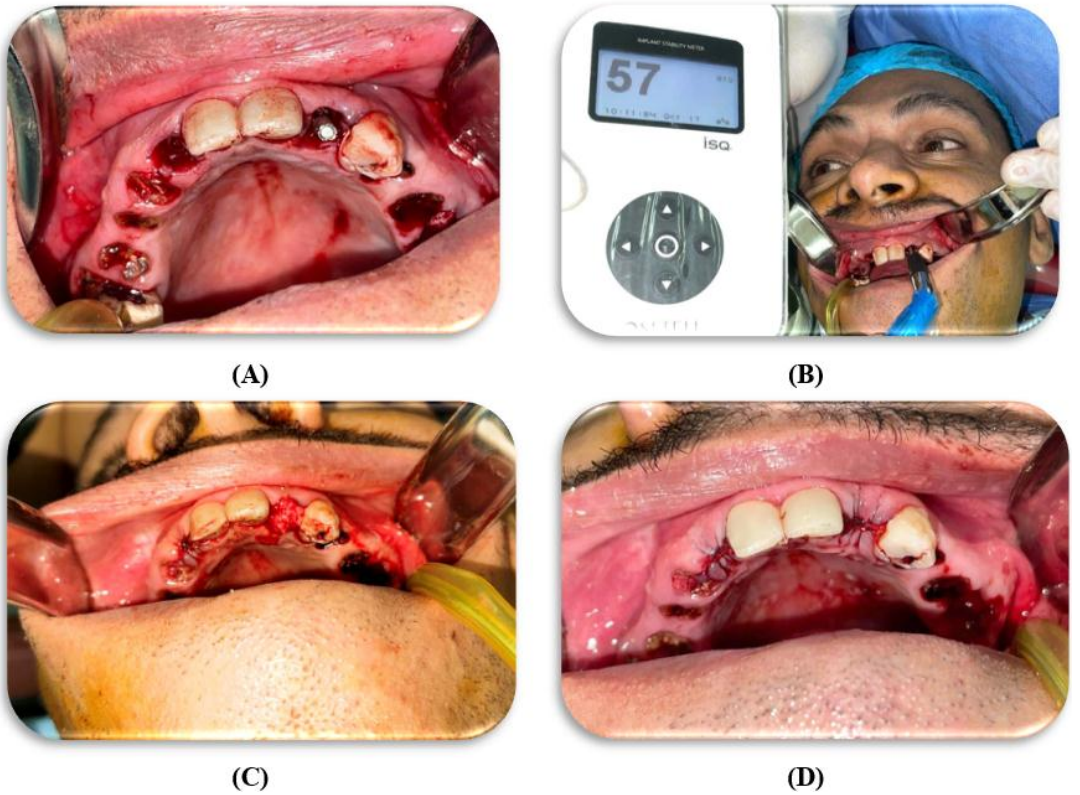


Figure 6: (A) Smart peg Inserted to the implant (B) osstell at implant placement (C) sticky autogenous fresh tooth graft with PRF (D) approximation and suturing of labial and palatal soft tissue

In group(2) The involved tooth was luxated using Periotome and small straight elevator. The root was extracted using remaining root forceps. Drills were used in the sequential manner to prepare implant site and extended 3 mm down the apex of the extracted tooth (Figure 7). The implant was inserted in the osteotomy site with the same manner as aforementioned in first group (Figure 8).

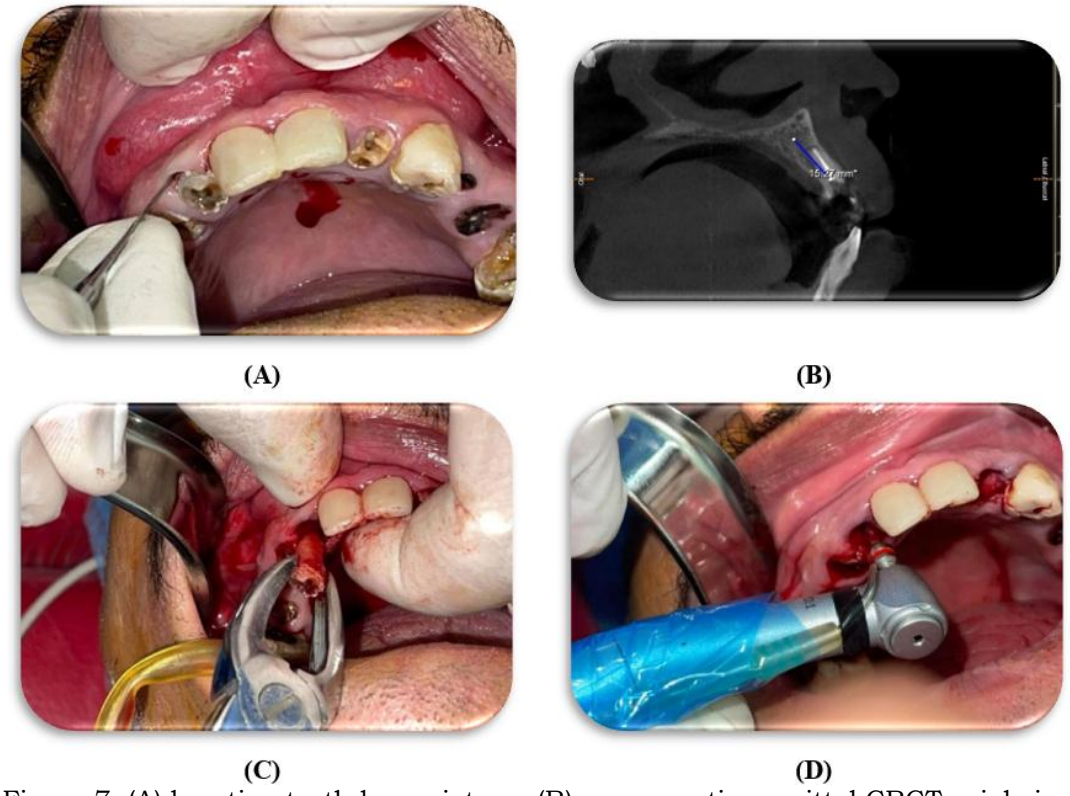


Figure 7: (A) luxating teeth by periotome (B) pre-operative sagittal CBCT axial view (C) extraction of remaining root (D) drilling of the Oeteotomy site

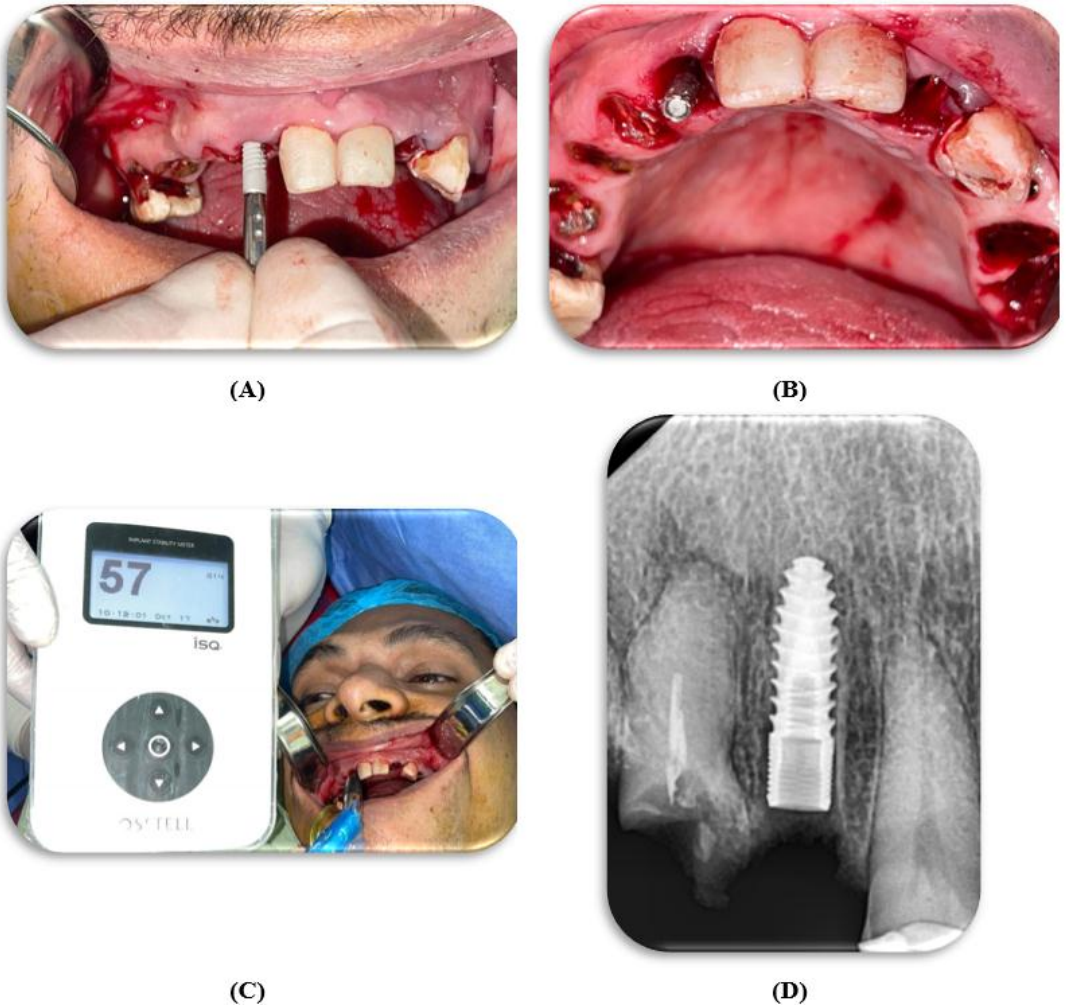


Figure 8: (A) placing of implant (B) placing of Magnatic peg (C) stability by OSSTELL (D) immediate post operative peri-apical x-ray

C-Postoperative phase

On the first day, all patients were asked to use cold packs extra orally sporadically every 10 minutes for two hours. On the second post-operative day, chlorohexidine mouthwash was started for one week, and the sutures were extracted one week after. 875 mg amoxicillin/125 mg clavulanic acid antibiotic (Augmentin 1 gm, GlaxoSmithKline, Australia), one tablet every 12 hours for a period of 5 days, was prescribed postoperatively as well as non-steroidal anti-inflammatory medication diclofenac sodium 50 mg tablets (Cataflam, Novartis pharma, Basel, Switzerland) five days, one tablet every eight hours . Chymotrypsin + trypsin® tablets (Alphintern, Kahira. pharm & chem. Ind. co., Cairo, Egypt), was administrated half an hour before meals 3 times for 7 days.

D- Follow up
Clinical evaluation

Following implant insertion, daily monitoring was maintained for the first week and then once weekly during first month for any symptoms of infection, discomfort, edema, or other post-operative problems.

Clinical examinations were conducted on patients for

The Visual Analogue Scale was used to assess pain. A score of 0 indicated no discomfort, while a score of 10 indicated the most severe excruciating pain. Postoperative problems were defined as the presence of discomfort, soreness, infection, or swelling, all of which might suggest the existence of peri-implant disease and potentially accelerated bone loss. Any complications that occurred postoperatively were documented. At four and six months following surgery, long-term follow-ups were undertaken to assess periodontal and gingival condition as well as stability of the implant.

Patients were evaluated clinically for

Probing depth peri-implant: the distance between the gingival margin and the buccal, palatal, mesial, and distal crestal bone borders. The mesial and distal pockets were evaluated from the buccal aspect as near to the contact sites as feasible, while the lingual and facial pockets were assessed at the implant's midline. Osstell ™ was used to assess implant secondary stability six months following implant insertion.

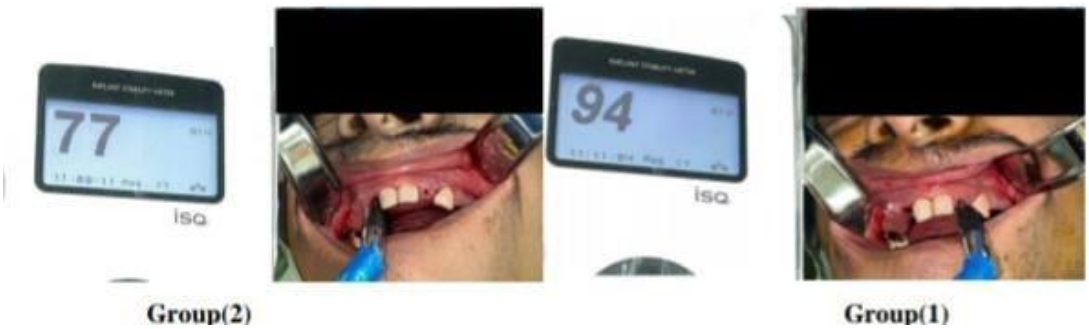


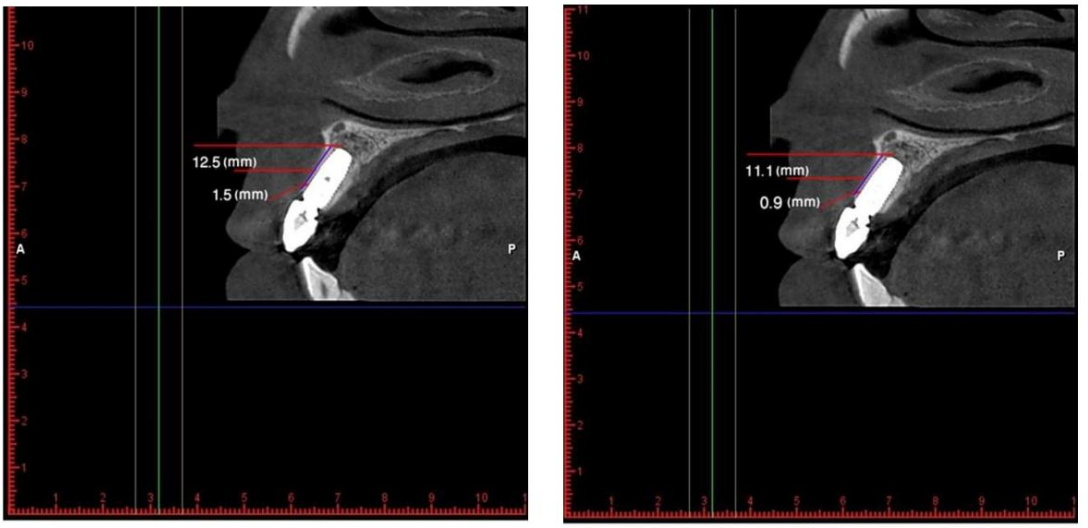
Figure 9: Implant stability quotient (ISQ) by OSSTELL at 6 months post-operative placement for groups (1) and (2)

Radiographic assessment

CBCT was used to measure horizontal and vertical dimensional changes in the labial bone following immediate implant insertion. This was done at 6 months.

Buccal bone width was determined using sagittal scans in the following manner

Horizontal bone level: A predetermined distance was drawn from the implant shoulder as a reference line, and the horizontal bone level was assessed during the follow-up period (Figure 10).



(A) **(B)**

Figure 10: (A) Photograph showing post-operative sagittal CBCT horizontal and vertical bone level in group (1) at 6 months (B) Photograph showing post-operative sagittal CBCT horizontal and vertical bone level in group(2) at 6 months.

Vertical bone level: A parallel line was drawn from the implant's apex to the CBCT's reference horizontal line, and the marginal bone level was determined from the reference line to the marginal bone crest parallel to the implant.

Statistical analysis

SPSS version 20 (IBM, Chicago, USA) The mean ± standard deviation of the data were used to represent them. The ANOVA test for repeated measurements was utilized to compare numerical variables among the patients in the study. If the ANOVA or Friedman tests were positive, a post hoc test was conducted. If the p-value was less than 0.05 in all tests, the results were declared statistically significant.

Results

12 implant fixtures were inserted in 6 patients divided equally into two groups (implant in each group). The male patients was 6 (50%) and female patients was 6 (50%). Each patient received two implants, one of them was tooth graft with PRF around immediate dental implant and other one without PRF. The age ranged from 20 to 35, with a mean value of 31.17 ± 6.05. All patients were operated on under local anesthetic, and no complications occurred during the procedure.

Clinical evaluation

All patients were assessed on a regular basis during the six-month follow-up period. In all cases, healing was uncomplicated, with no postoperative problems.
1**. Pain, swelling or infection;** All patients reported mild to moderate discomfort at the surgery site, which resolved entirely after the second and third days, and mild to moderate edema, which likewise resolved completely after five days. All patients remained infection-free during the follow-up period.
2. **Implant stability evaluation**; For group(1) At 3 months, the mean value of implant stability quotient was 82.83 ± 5.53 ISQ and At 6 months implant stability was increased to 90.67 ± 1.21 ISQ, which showed no statistically significant difference. For group(2) The mean value of implant stability quotient at 3 months was 70.50 ± 3.56 ISQ and increased to 77.67 ± 5.61 ISQ at 6 months, which was statistically significant. There was a statistically significant difference between the two groups (Table 1).

Radiographic evaluation

CBCT was used to measure vertical and horizontal dimensional alterations in the labial bone after maxillary anterior single instantaneous implant insertion in all patients.

1. Horizontal bone loss; For group(1) the mean value of horizontal bone loss at 6 months was 0.077(0.065-0.130), whereas the mean value of horizontal bone loss at 6 months for group(2) was 0.595(0.450-0.690). the difference in horizontal bone loss between two groups was statistically significant (Table 2).
2. Vertical bone loss; For group(1) the mean vertical bone loss at 6 months was 0.510(0.480-0.530), whereas the mean value of vertical bone loss at 6 months in group(2) was 1.490(1.400-1.640). the difference in vertical bone loss between the two groups was statistically significant (Table 3).

Discussion

Ridge alteration is a physiological process that must occur after either single or multiple teeth extraction, alveolar ridge undergoes resorption in both vertical and horizontal aspects [15]. Ridge loss starts after extraction and continues throughout life, its fastest rate during first three months, and then the rate decreases gradually [16]. Resorption occurs spontaneously in both horizontal and vertical dimensions, but it is more rapid and aggressive in horizontal dimension than in vertical one. Many studies stated that about 50% of the horizontal dimension was lost after six months vertical dimension decreases also, it decreases more rapidly on buccal side, after six months vertical dimension decreases by about 1.7mm [17].

To encourage new bone production, a variety of bone graft materials are employed. These include allografts, autogenous bone grafts, alloplastic grafts and xenografts. Due to their osteoinductive, osteogenic and osteoconductive qualities, as well as their ability to expedite recovery, autogenous bone transplants are considered the gold standard. However, autogenous bone transplants have some drawbacks, including a small graft area, resorption issues, and a second wound site infection. Additional graft materials such as allogenic, xenogenic, and

synthetic are employed. Allografts are less osteogenic and immunogenic than autogenous bone transplants, and they increase the risk of infectious disease transmission. Additionally, xenografts and alloplastic grafts exhibit greater osteoconductive properties and hence cannot contribute to desirable regeneration [18, 19].

Recently, grafts prepared from extracted teeth have been described as an alternative to other bone grafts in order to avoid the disadvantages associated with other grafts. This is because bone and tooth have a similar structure, as both originate from neural crest cells, and contain the same proportions of inorganic and organic components [7, 20].

In this study, autogenous tooth graft (ATG) absorption was slower than that of other grafts such as autogenous bone, xenograft, allograft, and alloplastic materials, owing to the fact that ATG was denser, which seems to be a typical issue with autogenous tooth transplants [21].

The advantages of ATG, which is highly osteogenic and does not have the disadvantages of autologous bone, suggest that it may be a viable alternative for patients who are allergic to allogenic or xenogenic graft materials. Another advantage of ATG over autologous bone harvesting procedures is that there is no donor site comorbidity, which may significantly reduce post-operative complaints and complications. Additionally, the clinician is not dependent on prefabricated bone grafts [22].

Demineralized dentin was used as graft material, where the extracted tooth was sent to Korea Tooth Bank® to be crushed and subjected to a dehydration, defatting and demineralization processes and then lyophilized, then sterilized with ethylene oxide gas, then sent back to the clinic or hospital, this process takes days to weeks to be done [23, 24]. A more recent device (VacuaSonic®) used to produce demineralized dentin graft chairside, but the process takes minimum of two hours, which is sometimes not applicable or acceptable by patients [25].

In the present study, the tooth extracted was grinded using Smart Dentin Grinder®(SDG) and sterilized by dentin cleanser then washed twice by phosphate buffered saline(PBS) to be ready for grafting within 15-20 minutes. SDG saving time, money and eliminating the need for second operation or waiting for a long time before grafting.

Platelet rich fibrin (PRF) is a second-generation platelet concentrate that promotes soft and hard tissue repair. It is composed of a fibrin network. The fibrin network contains stem cells and has an effect on the healing process's vascularization and angiogenesis. By tying the graft particles together, PRF provides mechanical stability. Additionally, PRF includes various growth factors that aid in the healing process. Autogenous PRF combined with a tooth transplant has been shown to be useful in a variety of areas of periodontal and bone surgery, including bone defect repair, periodontal treatment and sinus floor augmentation [26].

The current study investigated the use of tooth grafts in conjunction with PRF to augment the mean of the bone volume as well as the radiographical density. The

present study was in agreement with Ezgi YC, et al [27]. The presence of new bone formation in tooth graft mixtures containing PRF is critical for osteoblast differentiation and early bone healing. When considering the physiological stages of bone healing following tooth extraction, it is known that osteoblasts collaborate with fibroblasts to form a callus-like texture; this soft callus tissue is then mineralized to form mature bone tissue. Thus, a high rate of connective tissue creation in the group (1) after three months may be interpreted as a sign that new bone production continues [24, 28].

The rate of new bone and vessel formation was evaluated histopathologically around autogenous tooth grafts. Dense mesh fibrillar formations were observed in the tooth graft mixture with PRF group that were not seen in group (2), as well as the trabecular structure of the recently developed bone tissue and nonresorbed tooth particles [27]. Dense fibrillar structures may function as a scaffold between trabecula and tooth particles, hence exhibiting osteoconducting capabilities [29]. Additionally, it was discovered that the autogenous tooth transplant and bone cells communicated and that new bone grew immediately on the surface of the tooth graft particles [30]. Among the advantages of using PRF with autogenous tooth graft is increase the stabilization of graft particles [26].

For implant stability quotient (ISQ), the mean value of ISQ at 3&6 months respectively of group(2) showed decrease in ISQ with significant differences when compared to that in group(1) at 3&6 months respectively. PRF can significantly improve implant stability and give good tissue acceptance and biocompatibility, the present study was in agreement with the study of Qu C, et al [31].

Since the late 1980s, the implant-induced bone loss have been less than 1.5 mm in the first year following implant loading and less than 0.2 mm in subsequent years. Other studies have reported a mean crestal bone loss of 0.6 mm in the first year and 0.2 mm in the subsequent years up to 36 months following implant loading [32, 33], these studies show significant variability in marginal bone loss following dental implants.

In the present study the mean value of marginal bone loss at 6 months of group(2) showed increase in vertical bone loss with significant differences when compared to that in group(1) at 6 months. Marginal bone loss is likely to be decreased in group (1) owing to the use of rough-surfaced implants, which enhance the contact area between the implant surface and newly created bone. Additionally, tapered implants allow for improved stress distribution at the marginal bone implant interface [34], the present study was in agreement with the study of Hartlev J , et al [35].

The increase of bone density in group(1) indicates effective new bone formation, mineralization, remodeling, and maturation at the grafted site, as well as an improvement in peri-implant bone architecture and mineralization, which contributes to the implant's primary stability and osseointegration [36]. New bone within graft particles, slow resorption rate results of tooth graft in remaining graft particles with high density of graft. The present study was in agreement with the study of Kizildag A, et al [26].

Conclusions

Fresh autogenous tooth grafts may be an alternative graft material that overcomes the drawbacks associated with standard graft materials. The use of fresh autogenous tooth grafts in conjunction with PRF enhances bone formation capability and provides reliable clinical and radiographic indications of bone development and accelerated healing. Clinical follow-up over an extended period of time is necessary to assess the long-term bone development and survival rates of dental implants placed in grafted locations. Additional clinical investigations are required to evaluate the potential of autogenous tooth grafts to produce new bone with other graft materials.

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Conflict of Interest: Nil
Funding: Nil

Ethical approval: The study was conducted after obtaining the approval of the local ethical committee

Informed consent: Written informed consent was obtained from all patients and/or families

Author's contributions

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5
Concepts	+	+	+		+
Design	+	+	+	+	+
Definition of intellectual content	+			+	+
Literature search	+	+	+	+	
Clinical studies	+	+	+	+	
Experimental studies	+		+		
Data acquisition	+	+	+	+	+
Data analysis	+	+		+	+
Statistical analysis	+				
Manuscript preparation	+	+	+	+	+
Manuscript editing	+	+	+	+	
Manuscript review	+	+	+	+	+

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Tables

Table (1)
Comparison of Implant stability quotient (ISQ) between Immediate post-operative placement, 3 months and 6 months within group (1) and group(2)

	Immediate post-operative placement	At 3 months	At 6 months	Test used	P
Group(1) (n= 6)	54.83±9.79	82.83±5.53	90.67±1.21	F=47.17	**0.002***
Post-hoc		**P1=0.009***	**P2=0.001*** P3=0.08		
Group(2) (n= 6)	61.83±11.41	70.50±3.56	77.67±5.61	F=8.302	**0.038***
Post-hoc		P1=0.17	**P2=0.03*** **P3=0.04***		

Data expressed as mean±SD, SD: standard deviation, P:Probability, *:significance <0.05, Test used: Repeated measures ANOVA followed by post-hoc Bonferroni, P1: significance between Immediate & After 3 months, P2: significance between Immediate & After 6 months, P3: significance between After 3 months & After 6 months

Table (2)
Comparison of horizontal bone loss between group (1) and group(2) at 6 months.

	At 6 months	Test used	P
Group(1) (n= 6)	0.077(0.065-0.130)	Z= -2.226	**0.026***
Group(2) (n= 6)	0.595(0.450-0.690)	Z= -2.201	**0.028***

Data expressed as median (IQR), IQR:interquartile range, P:Probability, *:significance <0.05, Test used: Wilcoxon signed rank test

Table (3)
Comparison of vertical bone loss between group (1) and group (2) at 6 months.

	At 6 months	Test used	P
Group(1) (n= 6)	0.510(0.480-0.530)	Z= -2.201	**0.028***
Group(2) (n= 6)	1.490(1.400-1.640)	Z= -2.207	**0.027***

Data expressed as median (IQR), IQR:interquartile range, P:Probability, *:significance <0.05, Test used: Wilcoxon signed rank test