MethodsX 10 (2023) 102208

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Surgical method for critical sized cranial defects in rat cranium

Sedat Odabas[a,b], Burak Derkuş[b,c], İbrahim Vargel[d,*], Altughan Cahit Vural[e,*]

[a] Ankara University, Faculty of Science, Department of Chemistry, Biomaterials and Tissue Engineering Laboratory (bteLAB), Turkey
[b] Interdisciplinary Research Unit for Advanced Materials (INTRAM), Ankara University, Ankara, Turkey
[c] Ankara University, Faculty of Science, Department of Chemistry, Stem Cell Research Laboratory (SCRLab), Ankara, Turkey
[d] Hacettepe University, Faculty of Medicine, Department of Plastic Reconstructive and Aesthetic Surgery, Ankara, Turkey
[e] Harbiye Mah. Abdi İpekci Cad. 3/3 Şişli, İstanbul, Turkey

ARTICLE INFO

Method name:
Creating a critical sized bone defect in rat model

Keywords:
Cranium
Critical size
Bone tissue engineering

ABSTRACT

Cranial tissue models are a widely used model to show the bone repair and the regeneration ability of candidate biomaterials for tissue engineering purposes. Until now, efficacy studies of different biomaterials for calvarial defect bone regeneration have been reported, generally in small animal models. This paper offers a versatile, reliable, and reproducible surgical method for creating a critical-sized cranial defect in rats including critical steps and tried-and-tested tips. The method proposed here,

- Shows a general procedure for in vivo cranial models.
- Provide an insight to restore bone tissue repair that may be used in combination with several tissue engineering strategies
- Is a crucial technique that may guide in vivo bone tissue engineering.

Specifications Table

Subject Area:	Medicine and Dentistry
More specific subject area:	Tissue Engineering
Method name:	Creating a critical sized bone defect in rat model
Name and reference of original method:	Cranial bone regeneration via BMP-2 encoding mesenchymal stem cells doi:10.3109/21691401.2016.1160918
Resource availability:	Standard surgical operation equipment's were used

Introduction

Trauma, congenital deformations, or tumor resections may all result in cranial bone defects. Autologous bone grafts continue to be the gold standard for reconstruction. However, limited source availability and donor site morbidity are still the main limitations in use [1–3]. Allografts can be used as an alternative to autografts, but they also have some drawbacks, such as immunogenicity, a minor risk of disease transmission, and a relatively high cost of use [2–4]. In the US, nearly 3 million people suffer from traumatic brain injury after traffic or sports accidents. In some of these situations, bone reconstruction may be necessary to repair the bone defect and prevent further damage to the soft tissue underlying [5]. Generally, a proper biomaterial may be used to replace the missing or damaged piece of the skull. Yet, there is no single biomaterial has been shown to be superior to the others as the choice of the

* Corresponding authors.
E-mail addresses: ivargel@gmail.com (İ. Vargel), cahitvural@gmail.com (A.C. Vural).

https://doi.org/10.1016/j.mex.2023.102208
Received 2 December 2022; Accepted 3 May 2023
Available online 4 May 2023

materials for cranial reconstruction is strongly influenced by the clinical picture of the patient, the characteristics of the injury, the availability and cost of the biomaterial, and surgeon's choices [2]. Therefore, researchers have tried to find alternative biomaterials in the light of tissue engineering and regenerative medicine approaches [6,7]. Regulations such as the European MDR (medical device regulations) demand randomized clinical trials (RCT) to present the characteristics, biological compatibility, and regeneration ability of the material to be used in patients' needs for cranioplasty [7]. Before any clinical trials, the regenerative potential of a candidate biomaterial can be demonstrated over a cranial defect in a well-established critical sized bone defect. The critical-sized defect (CSD) for bone means that bone tissue will not heal spontaneously in a certain period of time and in the absence of any additional interventions. Over the past 30 years, researchers have tried to describe and present the experimental CSD approaches in different animal models [8–10].

However, there are still some controversies about defining the critical size. For cranial defects, convenient animals may be rodents such as rats, rabbits, and guinea pigs; however, the defect size may vary between species depending on the skull anatomy, healing, and regeneration mechanism of the animal. For instance, in laboratory rabbits, 15 mm cranial defect may be referred as CSD and in laboratory rats, above 6 mm cranium defect may be considered as a critical-sized cranial defect [9–12].

Here, we present a general procedure to create and operate on a critical-sized cranium defect in rats.

Materials and method

Animals

Male, preferably Wistar-Albino or Sprague-Dawley adult rats, weighing between 250 and 300 g (8 to 14 weeks old) [13]. The animals house in temperature and humidity-controlled rooms with a 12-hour light/dark cycle with free access to food and water ad libitum.

Reagents

- Sterile Saline Solution (Polifarma, Turkey)
- Povidone iodine (Konix, Turkey)
- Ketamin HCl (Alfasan, the Netherlands)
- Xylazine (Bioveta, Czechia)
- Terramycin gel (Pfizer, US)
- Ethyl alcohol,%96 (Merck, cat no: 159010)

Equipment

- Operation Table with Medical Examination Lamp Unit
- Rotary round-headed saw / trephine drill (Stoma, Germany)
- Animal Weighing Scale (Kent, US)
- Pre-sterilized Surgical Set (contains a scalpel, hemostats, scissors, forceps, etc.)
- #15 Surgical Blade
- Razor Blade or Shaver
- Nitrile Gloves
- Sterile fenestrated drapes
- Sterile Nitrile Gloves
- Sterile Syringes (1–10 mL)
- Sterile Gauze Pads
- Sterile Sample Containers (50 mL)
- Sterile Sutures (Silk 4.0, Braided Non Absorbable Surgical Suture)

Procedure

1. Always wear gloves before any approach.
2. Prepare a fresh anesthetic, including the combination of ketamine HCl (50 mg/mL) and xylazine (2%), considering the age and body weight of the animal. 50–75 mg/kg ketamine + 7–10 mg/kg xylazine may be used intraperitoneally for a proper anesthesia last 30 min. Tips: Always use fresh anesthetic cocktail. If you use the average weights of your animals to prepare the cocktail, please consider that small changes make vital effects on the maintenance and recovery of the animal.
3. Fill a 10 ml syringe with sterile saline solution.
4. Weigh the animal. Deliver the anesthetic solution intra-peritoneally and transfer the rats to a new cage.
5. Shave and clean the surgical area (cranium) before the operation. Tips: Generally, cleaning with 10% Povidone-Iodine solution helps disinfection of the area.
6. Transfer the rat to the operation table.
7. Disinfect the cranium by using a 10% Povidone-Iodine solution. Cover the surgical site with a sterile drape.

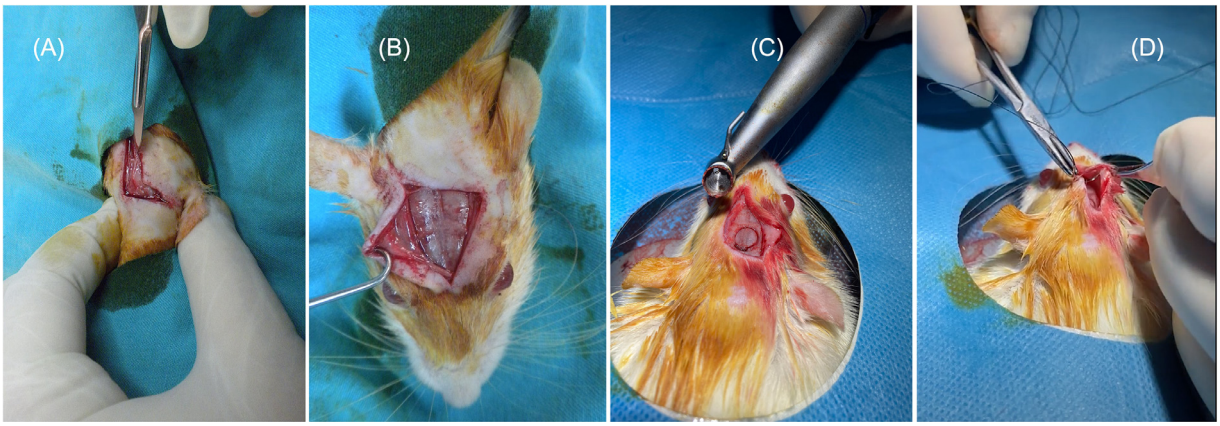

Fig. 1. Basic steps for performing a cranial defect in rat model. (A) small midline arcuate incision, (B) elevating the soft tissues, (C) Creating the defect with the drill, (D) Suturing the surgical defect.

8. Discard your gloves and wear a sterile pair of gloves and follow all aseptic surgical principles.
9. Create a small midline arcuate incision (approximately 2 cm long) from the nasal bone to the crest by using the #15 surgical blade (Fig. 1A).
10. Carefully elevate all soft tissues to reach the cranium. Note that the periosteum should be kept intact to keep body's own osteogenic induction (Fig. 1B).
11. Create a 6–8 mm craniotomy defect in the parietal bone by using a trephine drill Note that a continuous saline solution flush should be applied to prevent tissue burn (Fig.1C). Tips: Dura mater plays a key role in bone regeneration. Therefore, it is extremely crucial to keep the dura mater and sagittal sinus intact. Use the drill very gently and do not forget to flush a saline solution to the area. Do not attempt to completely remove the bone by drilling as over penetration may damage the underlying dura and the brain tissues.
12. Take out the cranial bone from the surgical site and graft the defect with a test sample (Name as Experimental Group); re-implant the removed bone tissue (Name as Autograft) or keep the defect empty (Name as Sham). Tips: Do not use sharp instruments to remove the bone, as these may damage surrounding and underlying soft tissues.
13. Close the craniotomy defect with a surgical suture according to common suturing techniques (Fig. 1D). Tips: The appropriate selection of suture size and needle edge type is crucial for a better tissue closure. The selection depends on several factors such as, tissue type, the need for removing the stitches, handling comfort, surgeon's experience and preference.
14. Apply a small portion of terramycin gel to the sutured region to prevent further microbial contamination.
15. Transfer the animal to a new cage and monitor all animals until the anesthetic effect wears off.
16. Provide adequate pain relief if needed.
17. Monitor the animal's overall health and welfare. Observe and record any signs of infection or other complications.

Method validation

Tissue engineering has the potential to be a game changer in the regeneration and reconstruction of bone defects. Over the past decades, several studies have been reported and presented an approach or a biomaterial to be used for the regeneration of cranial bone defects [6,7,14]. Moreover, there are also protocols to describe the way for creating cranial defects [15]. For a rat model, the best way to create a cranial defect is to use a dental trephine or drill. When the bone is excised, desired bone regeneration strategy can be applied. Previously, we demonstrated cranial bone healing either by using BMP-2-expressing cells or in combination with an osteoconductive scaffold, or even with scaffolds alone [16–19]. If the treatment will not have a systemic effect, our model may allow two groups per animal to be investigated. Thus, fewer animals can be used for the study. Some published protocols use isoflurane, a volatile halogenated anesthetic, to anesthetize the animals. However, there may be risks both for animals and individuals like researchers who are exposed to isoflurane, such as airway irritations, respiratory depressions, headaches, etc. [20,21]. Animals can be easily and carelessly overdosed while inhaling isoflurane. Therefore, using anesthetic gasses needs more technical infrastructure, calibration of the instruments, and technical use, and much care should be taken to use and discard waste them safely.

The protocols given here offer a ketamine/xylazine combination, which is a reliable anesthetic for small animal experiments and much safer than isoflurane, it may also give approximately 30-minute anesthesia for any interventions. The protocol here presents a facile procedure that may easily be applied within 20 min. Ketamine also helps reduce the use of painkillers during post-operative monitoring [22,23]. We also prefer non-absorbable silk, braided sutures for tissue closure, as silk sutures are comfortable and easy to handle, and they have good tension and knot-tying properties. These advanced knot-tying properties help in post-operation follow-up while rats often attempt to remove any stitches in their own or others skin.

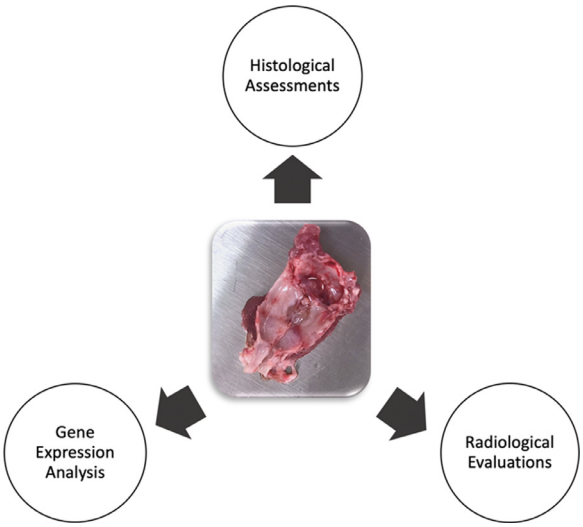

Fig. 2. Possible assessments of cranial bone samples.

The follow-up period after the operation may last a wide range of time periods, generally 1 to 12 months. Evaluations can be performed in consecutive months or in pre-determined periods [9]. It should be noted that sufficient bone regeneration and the associated development might not be observed in early follow-ups, and on the contrary, prolong follow-ups may lead unfavorable spontaneous healing in control groups. To assess the bone regeneration on the cranium defect size, certain evaluations should be performed after the animals are euthanized and the samples are removed from the implant site (Fig. 2) [16–18]. The basic evaluation is histological staining such as Hematoxylin-eosin and Masson's-trichrome. Moreover, a histomorphometry analysis can also be performed to score the bone regeneration and correlate the results Moreover, a micro-computed tomography (microCT) may also be used to quantify the new bone formation and the performance of the regeneration at the defect site. To understand the gene regulated bone healing and regeneration mechanism of protein synthesis, bone repair and regeneration related gene expression levels such as collagen I (COL1A1), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), osteonectin (OSN), osteocalcin (OSC), etc. can also be analyzed.

To conclude, we here present a routine practice for researchers to create a critical-size cranium defect in a rat model. Moreover, this procedure may be adapted and modified for other experimental animals with slight modifications. It should also be noted that researchers must follow ethical 3R's principles and conduct appropriate surgical techniques and post-operative care to maximize animal welfare.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sedat Odabas: Methodology, Writing – review & editing, Writing – original draft. **Burak Derkuş:** Methodology, Data curation, Visualization. **İbrahim Vargel:** Methodology, Data curation, Visualization. **Altughan Cahit Vural:** Methodology, Writing – review & editing, Writing – original draft.

Data availability

Data will be made available on request.

References

[1] H.C. Pape, A. Evans, P. Kobbe, Autologous bone graft: properties and techniques, J. Orthop. Trauma 24 (2010) S36–S40, doi:10.1097/BOT.0b013e3181cec4a1.
[2] H. Meyer, S.I. Khalid, A.H. Dorafshar, R.W. Byrne, The materials utilized in cranial reconstruction: past, current, and future, Plast. Surg. 29 (2021) 184–196, doi:10.1177/2292550320928560.
[3] R.R. Betz, Limitations of autograft and allograft: new synthetic solutions, Orthopedics 25 (2002) S561–S570, doi:10.3928/0147-7447-20020502-04.
[4] P.K. Moy, T. Aghaloo, Risk factors in bone augmentation procedures, Periodontology 81 (2019) 76–90, doi:10.1111/prd.12285.
[5] Cranial Fixation and Stabilization Devices Market Size, Share & Trends Analysis Report By Product (Fixation Devices, Stabilization Devices), By End Use (Hospitals, ASCs), And Segment Forecasts, 2018–2025; Report ID: GVR-2-68038-542-7, https://www.grandviewresearch.com/industry-analysis/cranial-implants-market
[6] H. Qu, H. Fu, Z. Han, Y. Sun, Biomaterials for bone tissue engineering scaffolds: a review, RSC Adv. 45 (2019) 26252–26262, doi:10.1039/C9RA05214C.

[7] S.E. van de Vijfeijken. Clinical relevance of current materials for cranial implants: towards an optimal patient-specific implant material, (2019) ISBN: 9789463324915.
[8] J.P. Schmitz, Z. Schwartz, J.O. Hollinger, B.D. Boyan, Characterization of rat calvarial nonunion defects, Cells Tissues Organs 138 (1990) 185–192, doi:10.1159/000146937.
[9] A. Vajgel, N. Mardas, B.C. Farias, A. Petrie, R. Cimões, N. Donos, A systematic review on the critical size defect model, Clin Oral Implants Res 25 (2014) 879–893, doi:10.1111/clr.12194.
[10] P.S. Gomes, M.H. Fernandes, Rodent models in bone-related research: the relevance of calvarial defects in the assessment of bone regeneration strategies, Lab. Anim. 45 (2011) 14–24, doi:10.1258/la.2010.010085.
[11] J.P. Schmitz, J.O. Hollinger, The critical size defect as an experimental model for craniomandibulofacial nonunions, Clin. Orthop. Relat. Res. 205 (1986) 299–308.
[12] E.H. Schemitsch, Size matters: defining critical in bone defect size!, J. Orthop. Trauma 31 (2017) S20–S22, doi:10.1097/BOT.0000000000000978.
[13] M. Hudieb, A. Haddad, M. Bakeer, A. Alkhazaaleh, M. AlKhader, D. Taani, S. Kasugai, Influence of age on calvarial critical size defect dimensions: a radiographic and histological study, J. Craniofac. Surg. 32 (2021) 2896–2900, doi:10.1097/SCS.0000000000007690.
[14] X. Wu, et al., Mineralized hydrogels induce bone regeneration in critical size cranial defects, Healthcare Mater. 10 (2021) 2001101, doi:10.1002/adhm.202001101.
[15] P.P. Spicer, J.D. Kretlow, S. Young, J.A. Jansen, F.K. Kasper, A.G. Mikos, Evaluation of bone regeneration using the rat critical size calvarial defect, Nat. Protoc. 7 (2012) 1918–1929, doi:10.1038/nprot.2012.113.
[16] A.C. Vural, S. Odabas, P. Korkusuz, A.S. Yar Sağlam, E. Bilgiç, T. Çavuşoğlu, E. Piskin, İ. Vargel, Cranial bone regeneration via BMP-2 encoding mesenchymal stem cells, Artif. Cells Nanomed. Biotechnol. 45 (2017) 544–550, doi:10.3109/21691401.2016.1160918.
[17] I. Inci, S. Odabas, I. Vargel, E. Guzel, P. Korkusuz, T. Cavusoglu, H. Kirsebom, E. Piskin, Gelatin-hydroxyapatite cryogels with bone morphogenetic protein-2 and transforming growth factor beta-1 for calvarial defects, Biomater. Tissue Eng. 4 (2014) 624–631, doi:10.1166/jbt.2014.1210.
[18] H.M. Aydin, P. Korkusuz, I. Vargel, E. Kılıç, E. Güzel, T. Çavuşoğlu, D. Uçkan, E. Pişkin, A 6-month in vivo study of polymer/mesenchymal stem cell constructs for cranial defects, J. Bioact. Compat. Polym. 26 (2011) 207–221, doi:10.1177/0883911511399411.
[19] İ.A. İşoğlu, N. Bölgen, P. Korkusuz, İ. Vargel, H.H. Çelik, E. Kılıç, E. Güzel, T. Çavuşoğlu, D. Uçkan, E. Pişkin, Stem cells combined 3D electrospun nanofibrous and macrochannelled matrices: a preliminary approach in repair of rat cranial bones, Artif. Cells Nanomed. Biotechnol. 47 (2019) 1094–1100, doi:10.1080/21691401.2019.1593850.
[20] Isoflurane Anesthetic Gas Guidelines, https://ehs.research.uiowa.edu/isoflurane-anesthetic-gas-guidelines, (Access: March 2023).
[21] E.P. Steffey, K.R. Mama, R.J. Brosnan, Inhalation anesthetics, in: Veterinary Anesthesia and analgesia: The Fifth Edition of Lumb and Jones, 2015, pp. 297–331. ISBN: 978-1-118-52623-1.
[22] V. Minville, O. Fourcade, J.P. Girolami, I. Tack, Opioid-induced hyperalgesia in a mice model of orthopaedic pain: preventive effect of ketamine, British J. Anaesth. 104 (2010) 231–238, doi:10.1093/bja/aeq075.
[23] M. Gao, D. Rejaei, H. Liu, Ketamine use in current clinical practice, Acta Pharmacol. Sin. 37 (2016) 865–872, doi:10.1038/aps.2016.5.