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INSTRUCTIONAL REVIEW - RESEARCH

The use of rats and mice as animal models in *ex vivo* bone growth and development studies

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In vivo **animal experimentation has been one of the cornerstones of biological and biomedical research, particularly in the field of clinical medicine and pharmaceuticals. The conventional *in vivo* model system is invariably associated with high production costs and strict ethical considerations. These limitations led to the evolution of an *ex vivo* model system which partially or completely surmounted some of the constraints faced in an *in vivo* model system. The *ex vivo* rodent bone culture system has been used to elucidate the understanding of skeletal physiology and pathophysiology for more than 90 years. This review attempts to provide a brief summary of the historical evolution of the rodent bone culture system with emphasis on the strengths and limitations of the model. It encompasses the frequency of use of rats and mice for *ex vivo* bone studies, nutritional requirements in *ex vivo* bone growth and emerging developments and technologies. This compilation of information could assist researchers in the field of regenerative medicine and bone tissue engineering towards a better understanding of skeletal growth and development for application in general clinical medicine.**

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Introduction

Globally, musculoskeletal disorders have a major influence on both the elderly human and animal.[1] It was estimated that 150 different musculoskeletal disease conditions affect both human and animal populations.[1,2] The most common conditions are those with greater economic impact, namely osteoarthritis, osteoporosis, rheumatoid arthritis, spinal disorders, limb trauma, gout, osteosarcoma, sprains and strains.[3] In the United States alone, the estimated total cost of these musculoskeletal conditions amounts to $250 billion annually.[3] The prevalence of these conditions and their economic burden have been projected to increase in future, in light of the ageing population.[4] There is thus a need for an in-depth investigation into the mechanism of and therapeutic measures for these conditions using different laboratory animal models.

In vivo and *ex vivo* animal studies have significantly contributed to numerous biomedical investigations into the discovery of drugs, clinical medicine, bone metabolism and morphological changes induced by mechanical loading of the bone.[5,6] These research areas had largely used animal models for the development and enhancement of numerous existing clinical therapies which involved soft and hard tissues in human and veterinary medicine.[7,8] *In vivo* animal model investigations are very costly and occasionally present with multiple challenges involving strict ethical considerations, problems associated with animal handling and alterations of normal physiological parameters due to fear and fright during experimental procedures. These challenges paved the way for multiple alternative *ex vivo* model systems.[9,10] In many ways, the *ex vivo* model system mimics the conventional *in vivo* model as the tissues and cells are morphologically positioned *in situ* within the normal extracellular matrix. This makes the *ex vivo* models in many biomedical investigations mimic the *in vivo* system.[11-13] Therefore, the *ex vivo* biology system could be a good substitute for some *in vivo* and *in vitro* models in an experimental design which is relatively cost effective and ethically acceptable in terms of animal welfare.[14-16]

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Table I. Summary of research involving animal species other than avian and rodent for *ex vivo* bone culture for bone growth- and development-related investigations[12,23-35].

Species involved	Types of bone used	Source
Canine	Cancellous bone	Rawlinson et al[23]
Bovine	Articular cartilage	Hall[24]
Ovine	Cancellous bone	Knothe Tate and Knothe[25]
Bovine	Trabecular bone	Smith et al[26]
Bovine	Articular cartilage	Bush and Hall[27]
Bovine	Articular cartilage	Bush and Hall[28]
Human	Trabecular bone	Smith et al[29]
Human, bovine and ovine	Trabecular bone	Davis et al[30]
Bovine	Trabecular bone	David et al[12]
Bovine	Articular cartilage	Amin et al[31]
Bovine	Wounded articular cartilage	Amin et al[32]
Bovine	Articular cartilage	Otsuki et al[33]
Bovine	Trabecular bone	Vinaco et al[34]
Human	Cortical femoral bone	Templeton et al[35]

The majority of the *ex vivo* bone developmental studies are intended for human application and other mammalian species using an embryonic chick bone model. However, there is a growth and developmental difference between avian and mammalian bone which could raise doubts in the interpretation of the experimental results for clinical application.[17] Farquharson and Jefferies,[18] and Nowlan et al,[19] reported that the secondary ossification centre of embryonic avian bone is absent before hatching, but does occur in mammalian fetal bone. The primary cartilage in fetal chick bones lacks vascular supply prior to mineralisation.[20] Other discrepancies between avian and mammalian bones include mammalian growth plate thickness which is relatively constant due to uniform blood supply and rate of bone resorption, as opposed to the irregular thickness of avian bone which is attributed to the absence of vascular supply to the primary cartilage.[18] In addition, the chondrocyte zones in the growth plate of the embryonic chick are not arranged in an orderly fashion compared with those of the mammalian species.[21] However, the cellular and molecular pathways of bone growth in the two species do occur in a similar manner. In view of these differences between bone growth in the two species, the use of avian embryonic chick bones for human and other mammals may raise doubt regarding its applicability. This review is a comprehensive attempt to report on the historical evolution of the rodent bone culture system, and on technologies that can assist researchers in the field of bone regenerative medicine and bone tissue engineering, and to understand the *ex vivo* skeletal growth and development of the model for application in general clinical medicine.

Evolution of the *ex vivo* bone culture system of bone growth. Numerous *ex vivo* models of bone growth and developments have been studied using different animal species, other than rodents. The majority of this research utilises pathogen-free chick embryos.[17,22] *Ex vivo* bone growth models and development studies, other than embryonic avian and rodent models, are summarised in Table I.[12,23-35]

The use of postnatal bovine cancellous bone for an *ex vivo* model appeared to be more popular compared with avian and rodent models. This could be due to the fact that bovine bone has true bone lamellar structure similar to that of human bone, which enhances interpretation and application in human studies. Trabecular bone is more frequently used than cortical bone because the former is metabolically active.[12]

The utilisation of *ex vivo* bone tissue culture for the study of skeletal growth and development was reported about 90 years ago using an embryonic chick model.[36,37] A subsequent study by Fell and Robinson[36] encountered some challenges in that the culture media then had to be supplemented with embryonic extract, plasma or serum. Culture media with such supplements were difficult to reproduce because the supplements were usually not produced in their purest form and contained variable concentrations of undesirable substances such as hormones. It is also difficult to differentiate between the cancellous or cortical bone growth requirements, as they differ in terms of their chemical matrices and as such respond to environmental changes independently.[12,17,14]

The aforementioned challenges triggered many investigators to conduct series of studies, particularly in the area of ideal standard media required for optimal *ex vivo* bone growth. Since then, numerous investigations have been conducted utilising embryonic chick bone models for skeletal growth and development.[21,38-44] In 1977, Messer[45] introduced a continuous-flow system of culture in an attempt to overcome the challenge of metabolite accumulation encountered in the stationary system. However, the method was not popular, possibly due to difficulties in reproducing and maintaining the exact experimental protocol.

Previously it was thought that embryonic bone could only be cultured *ex vivo* for a maximum of 18 days prior

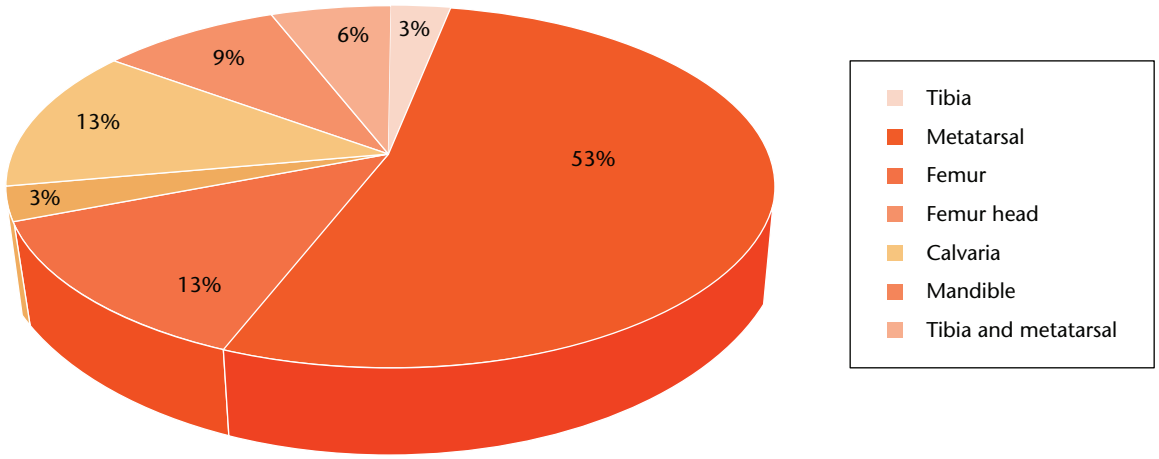

Fig. 1

Pie chart showing percentage distribution from the various species of rodent and the types of bones used for *ex vivo* bone growth studies based on the available studies published from 2000.

to bone tissue deterioration, based on the reported optimal culture period of ten to 18 days.[17] However, recent work conducted by Chagin et al[46] and Okubo et al[47] revealed that the embryonic bone of rats and mice could be cultured *ex vivo* for a longer duration of up to five or six months with the bone tissue remaining viable.

Rat and mouse *ex vivo* model for bone growth studies. The use of rats and mice as a model of bone growth started around the early 1960s,[48,49] exploring bone physiology using embryonic rat forelimbs at 19 days of gestation (E19) and mouse calvaria from five-day-old (P5) postnatal mice. *Ex vivo* bone culture studies involving bone physiology and the effect of hormones on bone is now common.[50,51] The early experiments documented only bone resorption, without evidence of bone formation.[48,52-54] Subsequent studies conducted by Bingham and Raisz[14] demonstrated that embryonic long bones of rats grew in length with evidence of new bone formation, which further showed that bone formation was influenced by the concentration of calcium, phosphate and magnesium ions in the culture media. This finding paves the way for numerous investigations into the nutritional requirements for optimal growth of bone in the *ex vivo* environment. Raisz[48] used Eagle's medium for standard cell culture which was supplemented with human serum, while Goldhaber[49] used Gey's balance salt solution supplemented with heated horse serum and chick embryo extract. Although the bones were cultured in a different microenvironment, successful physiological activities were demonstrated. Raisz[48] incubated the embryonic bones in a humidified plastic chamber for 72 hours in 5% CO_2, 20% O_2, and 75% N_2 at 37°C, while Goldhaber[49] incubated the bones in a humidified stationary culture tube containing 95% O_2 with 5% CO_2 for 12 days. Following these findings, many researchers developed an interest in bone tissue-related research, focusing attention on skeletal growth and development with an emphasis on the pathophysiology of different skeletal disease conditions.

Previous studies used large containers such as petri dishes, stationary tubes and Leighton tubes to incubate the bone.[14,48,49] The disadvantage of using petri dishes and tubes is that it requires a large volume of media to sufficiently cover the bones. Subsequent studies have overcome this by incubating the bone tissue in either six- or 24-well plates depending on the size of the bone intended to be cultured.[44,55-64] Currently, some studies involving bone culture *ex vivo* focus on the use of a dynamic bioreactor and chorioallantoic membrane (CAM) culture system.[22]

***Ex vivo* model for consideration: rat versus mouse.** Available research records that used rats and mice for *ex vivo* skeletal growth and development published since 2000 showed that both rats and mice have been equally used for *ex vivo* bone culture investigations. This could indicate that both species used for *ex vivo* bone culture studies probably give similar results when used as a model for bone growth. Other reasons that likely warrant the choice of either rat or mouse are the personal preference of the investigator or the protocol intended to be adopted. This trend could change in the future, with the possibility of a slight shift towards the use of more rat models. This is due to the advantage of the larger size of rat bones which can be easily dissected intact without morphological alteration of the bone structures. However, the murine model is gaining popularity because of the availability of genetic modification, i.e. the gene knockout model. With this in mind, the frequency of murine model usage may also likely be higher in comparison with that of rats in future investigations.

With regards to the type of bone used, available research shows that the metatarsal bone had the highest percentage (53%) of use when compared with other bone types (Fig. 1). The exact reason for this frequent usage is not known but it could be associated with the small size of the bone which can be easily and

conveniently cultured in relatively small culture plates. The other likely reason for the frequent use of metatarsal bone could be its greater numbers, i.e. five metatarsal bones per limb, which provides an adequate sample size for statistical analysis. The preference could also be attributed to the low degree of calcification in early postnatal life (days 1 to 7),[65] which facilitates histological processing without the need for decalcification for intact *in situ* histomorphological studies.

The choice of calvaria and femoral bones each represent 13% of the published data (Fig. 1). The preference for the femur, following the metatarsal, could be due to the former providing a good model for endochondral bone formation. Nevertheless, this has a major disadvantage from the point of view of its large size, which may require a huge volume of growth media in a conventional static bone growth set-up. However, the femur is the preferred *ex vivo* model for breast cancer metastasis because it is considered to be highly susceptible to most soft-tissue cancer metastases.[66,67] Stern and Krieger,[56] and Schwartz et al[66] reported that the choice of calvaria for *ex vivo* bone culture models provides an easy system for intramembranous bone growth. It also offers the additional advantage of providing enough material for a simple biochemical analysis. Recent studies conducted by Curtin et al[68] and Krishnan et al[69] also used neonatal and postnatal calvaria to study cancer metastasis.

Studies have also shown that the selection of neonatal and postnatal bones is almost equal to that of neonatal bone (53.1%), which indicates that the two stages of rodent (embryonic and post-embryonic) development have almost equal preference in *ex vivo* skeletal growth and development studies. Earlier research, prior to 2000, showed more frequent use of embryonic bones compared with postnatal bones,[17] The reason being that embryonic bone tissue has a higher capacity for bone growth and metabolic activities, as reported by Mohammad et al.[70]

Nutritional requirement of bone growth *ex vivo*. Early media used for *in vitro* cells and tissue growth were pieces of tissue explants, human or animal plasma or fibrinogen clots. The growing cells or tissues are attached to these biological substances. The media are supplemented with human placental serum, avian embryonic extracts or balanced salt solution.[71] Currently, there is a wide variety of commercially available media that have been tested to support the growth of both embryonic and postnatal bones in an *ex vivo* microenvironment. Commercially available media contain a range of 25 to 29 macro nutrients components made up of 13 amino acids, seven vitamins, glucose, and seven different salts with different types of minerals that support the growth of bone *ex vivo*.[72] The standard bone culture media are supplemented with various nutritional ingredients and antibiotics at the discretion of the researchers for optimal growth of bone and suppression of bacterial contaminants. Most commonly available supplements include bovine albumin and fetal calf serum, ascorbic acid, sodium glycerol phosphate, glutamine and many other supplements depending on the adopted protocol. Phenol red is usually added to the standard medium to indicate level of tissue utilisation and metabolic activities. The most widely used media for bone tissue culture include Dulbecco's modified Eagle medium (DMEM), alpha-minimum essential medium (α-MEM) and Biggers, Gwatkin and Judah (BGJb) medium. The DMEM and BGJb are modifications of Basal Medium Eagle (BME) while α-MEM is a modification of Harry Eagle's minimum essential medium (MEM or EMEM).

Current development in the field of bone culture systems. Currently, *ex vivo* bone growth and development can be assessed with the two latest models: the dynamic 3D bioreactor and the CAM culture system. The introduction of bioreactor techniques in animal experimentation has made it possible to conduct biomechanical force investigations within *in vitro* and *ex vivo* conditions.[12,73-75] It was observed that culturing of whole bone in the dynamic 3D bioreactor can enhance tissue perfusion during *ex vivo* growth;[12] this is achieved by ensuring uniform culture media perfusion into the bone tissue thereby permitting adequate delivery of nutrients into the bone core. The system also allows for the assessment of mechanical parameters of the cultured bone. Bone viability could be maintained for considerably longer with a maximum media perfusion flow rate of 6.6 mLh^{-1} under controlled biochemical and mechanical conditions.[34] The system permits monitoring of culture conditions such as pH, temperature, and the supply of oxygen and carbon dioxide. The culture media need to be changed every 24 to 48 hours as in conventional static bone culture systems.[30,34] One of the greatest advantages of the bioreactor for *ex vivo* bone culture, apart from improved culture media circulation, is the reduction in the number of handling steps, which may likely minimise potential contamination of the bone tissue.[76]

There are two types of bioreactors that are currently available for *ex vivo* bone culture,[77] namely the rotating oxygen-diffusing vessels and magnetic force bioreactors. The rotating oxygen-diffusion system is usually filled with culture media and perfusion systems incorporated to control the back-and-forth flow within the system. It was reported by Partap et al[78] and Rauh et al[79] that the rotating oxygen-diffusion bioreactor system has an increased osteogenic cell multiplication capacity, and enhanced osteogenesis. *In vitro*-enhanced differentiation and maturation of human mesenchymal stromal cells to chondrocytes was also observed by Hoffmann et al.[80] It was also reported that bone viability can be maintained for a longer duration which could translate to increased osteogenesis in cell-seeded bone when the rotating oxygen-diffusion

system is combined with the compression bioreactor that applied mechanical force on the bone tissue.[81-83] The magnetic force bioreactor applies forces directly to the cell membrane within the bone tissue, rather than to the surrounding bone scaffold. This task is accomplished by cell attachments to an in-built magnetic nanoparticle.[84,85] The system can also enhance expression of bone extracellular matrix proteins, and osteogenic cell differentiation and their proliferation.[76,86] It can also mechanically stimulate mesenchymal cell-seeded bone scaffolds, osteogenic cells and whole bone explants.[87-90]

Currently only human, bovine and ovine trabecular bones have been cultured using the dynamic bioreactor system in studies investigating the biomechanics of cancellous bone during growth, remodeling and modeling investigation trials.[12,85] Bioreactor technologies are yet to be fully adopted for use in rodent *ex vivo* bone culture. If adopted for bone growth and development, it may enable application of the *ex vivo* tissue culture system to be a closer approximation of the *in vivo* model outside the living environment. However, the utilisation of a bioreactor system has been initiated for the *ex vivo* culture of avian bone; a study was conducted by Henstock et al[91] using a customised bioreactor system that was developed mainly for culturing an embryonic chick femur. The results showed an increase in bone growth and mineralisation.

The CAM culture system is a new modification of the *ex vivo* tissues/organ culture system.[92-97] The model uses a minimum 72-hour-old embryonic egg in which a small window is made on the shell surface into the CAM. The tissue to be cultured will then be inoculated directly on the CAM next to the developing chick embryo.[95] The *ex vivo/ex ovo* culture system was found to be very useful for studies involving avian, human and rodent tissues in diverse fields of scientific investigation ranging from tissue angiogenesis,[98-101] anti-angiogenesis,[98,102] oncology,[103-106] obstetrics,[96,107,108] pharmaceuticals,[95,109,110] bone development,[111] imaging,[109,112] microsurgery,[112] culturing of xenotransplanted tissues and other biomedical applications.[108,113] In the area of bone tissue engineering research, the CAM system was successfully used to investigate tissue neovascularisation during bone graft, regeneration and critical bone size defect studies.[114-116] The CAM *ex vivo* culture systems have advantages over conventional static *ex vivo* systems of tissue culturing. The extra-embryonic membrane system has the capability to mediate oxygen and nutrient exchange within the chick embryo. The system was reported to be highly vascularised in such a way that it can be easily connected with the vascular network of implanted tissue. Above all, the model allows exogenous materials from other species to be implanted without immunogenic rejection as the embryonic chick immunity is usually not fully developed to challenge foreign bodies.[116-118] To date, the use of the CAM model system is grossly under used in rodent *ex vivo* bone culture investigations. If the CAM model is applied with meticulous care, it would go a long way to provide an alternative that is a simple, reliable and inexpensive *ex vivo* bone culture system. Recently, the CAM model has been used for chick bone culture studies.[17] The major drawback of the system is that *ex vivo* cultures cannot be carried out for a longer duration more than 15 to 18 days, as chick embryos develop within a limited period of approximately 21 days.

Reliability of the three *ex vivo* bone culture models. The 3D dynamic bioreactor bone organ culture was introduced for bone engineering applications to overcome challenges that were associated with conventional static bone culture systems. It was demonstrated to have improved distribution and to facilitate osteogenic cell maturation,[119] but the majority of the bioreactor systems for bone engineering at present were designed with a low volume output. Additionally, their assembly is usually time-consuming and may exhibit operator-dependent variability. Therefore, the conventional static *ex vivo* bone culture is still considered the most practicable, reliable, easy and inexpensive means of *in vitro* growth of rodent bone for study involving osteogenic cell behaviour in a controlled environment.

Generally, the reliability of all the *ex vivo* tissue/organ cultures depends on the provision of a conducive physiological atmosphere that replicates the normal body condition of the explants in an intact organism. Therefore, the reliability of the conventional static *ex vivo* bone culture model and that of the dynamic 3D bioreactor model depend on the ability of the osteogenic cells to be in the appropriate extracellular matrix (ECM) compartment of the bone tissue microenvironment and retain viability and metabolic activity

The ECM is one of the important components of the bone tissue microenvironment because its presence is valuable for the maintenance of the physiological function of osteogenic cell polarisation, survival and proliferation.[120] The whole bone tissue should be able to remain viable and continue to grow in the *ex vivo* culture environment for a minimum incubation period of 48 hours in a static culture system. In the 3D dynamic bioreactor system, bone cells should be able to maintain viability for an incubation period of at least 21 days.[34] The metabolic activity of osteogenic cells are measured by their mineral deposits, therefore measurable mineral apposition should be recorded similar to the *in vivo* response at different ages and specific to the species of the animal model used.

In addition to cell viability, for the bioreactor bone organ culture to be reliable, the cultured bone must be able to demonstrate a response to a mechanical stimulus.[121] In order to achieve reasonable growth of the whole bone in the dynamic bioreactor system, the osteogenic cells must undergo mechanical loading stimuli similar to those obtained in an *in vivo* scenario, as it was reported

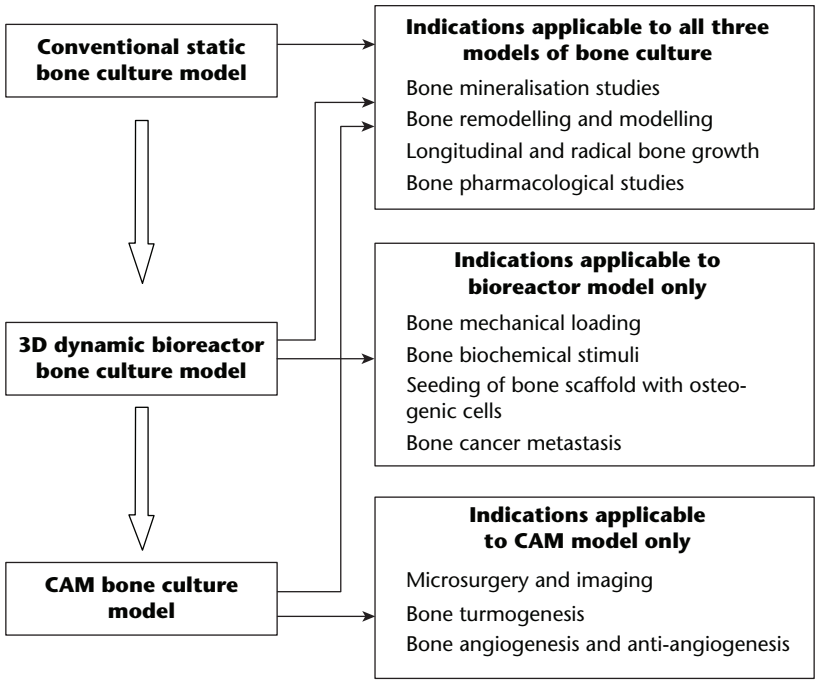

Fig. 2

Order of preclinical application of the *ex vivo* rodent bone culture models and their indications in bone growth, development and related fields of studies. Note that all indications that are applicable for the conventional static culture system are equally applicable to other models. The block arrows indicate the current order of the mostly used technique (from top to bottom) among the three *ex vivo* models reported (CAM, chorioallantoic membrane).

that during locomotion *in vivo*, the bone cells are believed to undergo oscillatory and pulsatile flow.[78]

In order to maintain osteogenic cell viability, all of the bioreactor types for *ex vivo* bone culture were designed to function based on forced media flow around the bone tissue to provide nutrient delivery deep into the bone core beyond the periosteal bone surfaces, increase oxygenation within the construct, and eliminate the metabolic waste accumulated within the system.[79] The fluid flow within the bioreactor system has also been shown to mechanically stimulate bone formation by increasing the osteogenic formation markers as reported by Batra et al,[122] Kreke et al,[123] and Jaasma and O'Brien.[121] The formation of the osteogenic markers was also reported to improve bone mineralisation, which could lead to increased mechanical strength of the bone.[124] It was also reported by Jaasma and O'Brien[121] that whole bone tissue cultured in a continuous low flow rate medium of 0.01 mL/minute resulted in a high proportion of viable bone cells and an enhanced osteogenic response with improved spatial distribution of osteogenic cells when compared with the static culture method.

The overall survival rate of the chick embryo in either the *in ovo* or *ex ovo* technique is the major concern in the CAM *ex vivo* organ culture system. Therefore, an appropriate conducive incubation environment is the first priority, followed by a strict aseptic procedure devoid of microbial contaminants in order to reduce the level of environmental contamination to the bare minimum.[98] It has been reported that about 25% to 50% of the chick embryos die after manipulation of the egg.[103,110]

The reliability of the CAM *ex vivo* organ culture depends largely on the formation of new vascular networks in the implanted tissue, which may occur within a minimum of 48 hours after culture as reported by Dohle et al.[94] The neovascular anastomosis can be checked and confirmed by various image analyses at different intervals during the incubation period. Quantitative vascular density can be evaluated using vesicular endothelial proliferation techniques.[99]

Strength, indications and limitation of *ex vivo* bone culture models. The rodent *ex vivo* model system for bone growth has contributed significantly to scientific progress in the fields of developmental biology, genetics, cancer biology, cell biology and tissue engineering. The rapid development of rat and mouse skeletal tissue adaptation for *ex vivo* culture has become almost as popular as the avian model system which is extensively used in the field of skeletal biology studies.[8] The *ex vivo* culture system is relatively cost-considerate; easily manipulated, and rapidly develops multiple cell types in their natural matrix.[64]

At present, the conventional static bone culture model is still the most widely used system for *ex vivo* bone culture for preclinical investigations in the field of musculoskeletal investigations. The order of most commonly reported models as discussed in this review is shown in Figure 2. The three different models are indicated for different fields of bone growth and developmental studies,

but there are common indications that are applicable for all models. The 3D bioreactor model appears to have wider applications, whereas the conventional static bone culture model has the most limited research applications.

The conventional static and the 3D bioreactor bone culture models have disadvantages such as deficiency of blood supply to the *ex vivo* setup which remains a serious setback, since angiogenesis is critical for nutrient supply within the bone tissue.[125-128] On the other hand, the lack of blood supply provides a unique opportunity to study cartilage and bone development and formation without being complicated by the vasculature.[129-130]

In conclusion, the rodent *ex vivo* bone culture system has great research potential in diverse medical fields. The system can be useful in understanding the complex biological events during bone growth development and fracture healing. It also provides a simple, inexpensive screening model with limited ethical considerations, and it addresses the issues of reduction, refinement and replacement (3 Rs) in an animal experimental system. These advantages of the rodent *ex vivo* model over conventional *in vitro* and *in vivo*, along with the developments of the dynamic bioreactor for *ex vivo* and CAM culture systems, will further enhance and improve the model. This also indicates that the *ex vivo* bone growth model will continue to provide a significant role in the growing field of skeletal growth and development in future.

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Author Contribution
- A. A. Abubakar: Initial draft, Editing, Data analysis, Reviewing the paper.
- M. M. Noordin: Reviewed and edited the paper.
- T. I. Azmi: Reviewed and edited the paper.
- U. Kaka: Initial draft, Editing, Data analysis.
- M. Y. Loqman: Initial concept, Participated in initial draft, Editing, Data analysis, Reviewing of the paper.

ICMJE conflict of interest
- None declared