Effects of ^{153}Sm-Ethylenediaminetetramethylene Phosphonate on Physeal and Articular Cartilage in Juvenile Rabbits

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Previous studies reported that the radiopharmaceutical ^{153}Sm-ethylenediaminetetramethylene phosphonate (^{153}Sm-EDTMP) is an effective component of multimodality therapy for the treatment of primary bone tumors. Therefore, ^{153}Sm-EDTMP may prove to be an integral component of therapy for the treatment of juvenile osteosarcoma. The purpose of this study was to determine the effects of intravenous administration of ^{153}Sm-EDTMP on the developing physeal and articular cartilage of healthy, juvenile rabbits. **Methods:** Sixteen healthy 8-wk-old male New Zealand White rabbits were assigned to 1 of 2 groups: treatment (n = 12) and control (n = 4). ^{153}Sm-EDTMP was administered to the treatment group at 37 MBq/kg (1 mCi/kg). The animals were sacrificed at 16 wk of age, and the physeal cartilage of multiple bones was evaluated by use of histologic, immunohistochemical, and histomorphometric analyses. The overall changes in the lengths of the radius and the tibia between control and treatment groups were calculated and compared. Measurement data were combined for each group, and means ± SEMs were determined. **Results:** Significant differences in radial bone growth were present between the groups. Histologically, the physes of the treatment group were disrupted and chaotic in appearance. Significant differences in the immunoreactivity of type X collagen and matrix metalloproteinase-13 were seen between the groups, as these markers were positively expressed in the zone of hypertrophy of the control rabbits. **Conclusion:** Clinically significant damage to the developing physeal cartilage may occur as a result of the intravenous administration of ^{153}Sm-EDTMP at the dose studied.

Key Words: growth; ^{153}Sm-ethylenediaminetetramethylene phosphonate; osteosarcoma; physis; rabbit

J Nucl Med 2003; 44:1510–1515

The effects of external beam radiation on growing physeal cartilage have been established (*1–6*), but the effects of the β-particle–emitting radionuclide ^{153}Sm-ethylenediaminetetramethylene phosphonate (EDTMP) on physeal development and growth plate closure have not been evaluated. In people, radionuclide therapy is used primarily for the relief of pain associated with bone metastases resulting from breast, lung, and prostate cancer. Its efficacy is well documented (*7–11*). Canine studies and limited studies in people have suggested that intravenous radionuclide therapy can be an effective component of a multimodality treatment regimen for primary bone tumors (*12–16*). Expansion of these studies may eventually lead to the use of this agent in juvenile osteosarcoma. The objective of the present study was to determine the effects of intravenously administered ^{153}Sm-EDTMP on physeal and articular cartilage development in rabbits. This work will serve as an initial study to determine the potential of this agent for causing developmental abnormalities because of its preferential deposition in areas with high osteoblastic activity.

^{153}Sm-EDTMP possesses physical properties that make it an ideal therapeutic radiopharmaceutical. The medium-energy β-particle (energy maximums of 0.640 MeV at 30%, 0.710 MeV at 50%, and 0.810 MeV at 20%) limits the deposition of ionizing radiation to a distance of about 2 mm from the point of decay (*12*). ^{153}Sm-EDTMP also emits a 0.103-MeV γ-photon at 28%, allowing the imaging of skeletal localization with conventional gamma cameras (*7,12*). ^{153}Sm is chelated to EDTMP. Upon intravenous injection, this complex localizes in areas of increased osteoblastic activity, and deposition of this agent is directly correlated to matrix formation. This process can occur in regions of bone growth and remodeling as well as tumor formation (*7,12*). Chelation to EDTMP is advantageous because it enhances the skeletal uptake of ^{153}Sm but markedly limits its deposition in soft tissues.

The potential for delivering a curative dose of radiation to bone tumors by combining an intravenously administered

Received Nov. 22, 2002; revision accepted Apr. 21, 2003.

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radiopharmaceutical with external beam therapy warrants further investigation for the possible treatment of primary bone tumors. The peak age of incidence for osteosarcoma is in the second decade of life. Patients may have not reached their adult stature, and insults to the physes by ionizing radiation could cause permanent cessation of growth or growth deformities and possibly predispose these patients to pathologic fractures. Because ^{153}Sm-EDTMP localizes in areas of high osteoblastic activity, such as the zone of provisional calcification, substantial injury to physeal and articular cartilage in normal bone may occur. We hypothesize that ^{153}Sm-EDTMP administered intravenously at a therapeutic dose will irradiate the physeal cartilage of juvenile rabbits, leading to growth abnormalities.

MATERIALS AND METHODS

Initial Preparation

This animal experiment was conducted in compliance with the regulations of the Animal Use and Care Committee of the University of Missouri. Sixteen juvenile (8 wk old) male New Zealand White rabbits (Myrtle Rabbit Tree) were randomly assigned to 1 of 2 groups: treatment ($n = 12$) and control ($n = 4$). On day 0, the rabbits were anesthetized, and marking wires were placed in the proximal and distal tibial diaphyses to serve as reference points for subsequent limb length measurements. Rabbits in the treatment group were given ^{153}Sm-EDTMP at 37 MBq/kg (University of Missouri-Columbia Research Reactor) intravenously. Rabbits in the control group received a sham injection. At 48 h after injection, whole-body scans were acquired with a gamma camera (Dyna-Camera IV; Picker International, Inc.) to assess radiopharmaceutical uptake (Fig. 1).

Lateral and craniocaudal radiographs of the tibias, radii, ulnas, and humeri of all rabbits were obtained 0, 2, 4, 6, and 8 wk after injection by use of standard equipment and techniques. This schedule was chosen to allow for a sufficient increase in the total length of each bone between each set of measurements. The rabbits were anesthetized for each radiographic procedure to ensure proper positioning. A linear standard was included in all radiographic views to permit correction for magnification. Measurements of total bone length, physeal thickness, and proximal and distal bone lengths were obtained for the tibia at each time point by use of a computer software program (Scion Image; Research Service Branch of the National Institutes of Health). All radiographs were captured as digital images by use of a computer software program (Photoshop; Adobe Systems Inc.) and magnified to 300% for accurate evaluation. The lateral, medial, and central aspects of the physes were each measured 3 times, and values were averaged to obtain the physeal thicknesses. Measurements of total bone lengths for the radii and tibias were also obtained at each time point. The length of each tibia was measured from the medial tibial condyle to the medial malleolus. The length of the radius was measured from the most proximal-medial aspect of the radial head to the styloid process of the radius. Radiographs of the tibias, radii, and humeri were subjectively evaluated for gross abnormalities, including angular limb deformities and evidence of premature growth plate closure. All rabbits were sacrificed 8 wk after injection.

FIGURE 1. Whole-body lateral scintigram of juvenile rabbit 48 h after administration of 37 MBq of ^{153}Sm-EDTMP. Increased degree of radionuclide uptake is located in physeal or metaphyseal area of long bones.

Histopathologic Evaluation

The tibias, radii, ulnas, and humeri of all rabbits were collected for histologic processing. Before harvesting of the bones, all soft tissues were removed. The specimens were placed in formalin and decalcified with hydrochloric acid and ethylenediaminetetraacetic acid in water (Decalcifier II; Surgipath Medical Industries, Inc.). After routine histologic processing, 5-μm sections were cut and stained with hematoxylin–eosin, toluidine blue, and periodic acid–Schiff stains by standard methods. All sections were subjectively evaluated for physeal and articular cartilage morphology and extracellular matrix (ECM) staining by 2 blinded investigators. Physes were subjectively scored as follows: open, organized; open, mildly disorganized; open, moderately disorganized; and closed. Histomorphometric analyses of all physes were performed as an objective assessment of physeal dimensions. Toluidine blue–stained slides were captured as digital images by use of Photoshop. The digital images were used to determine physeal width, height, and area by tracing the histologic perimeter of each physis and calculating each dimension with an image analysis program (ImagePro; Media Cybernetics). Each dimension was standardized to physeal length to normalize the data.

Immunohistochemical Evaluation

After routine histologic processing, sections from each sample were stained with monoclonal antibodies to type X collagen (mouse, antideer) (Gary Gibson, Henry Ford Hospital), matrix metalloproteinase-1 (MMP-1) (mouse, antihuman) (Ab-1 Oncogene Research Products), and MMP-13 (mouse, antihuman) (Ab-1 Oncogene Research Products) by use of an avidin–peroxidase immunohistochemical technique (type X collagen, 1:600 dilution; MMP-1, 1.0 μg/mL; and MMP-13, 2.0 μg/mL). Unstained 5-μm sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by immersion in 3% H_2O_2 in methanol. After antigen retrieval treatment by use of trypsin, nonspecific binding was blocked with normal blocking serum (100% goat serum). Slides were incubated overnight at 4°C. On the next day, the slides were incubated with biotinylated secondary antibody (LSAB 2 System; DAKO Corp.). Bound primary antibody was detected by use of an avidin–horseradish peroxidase method (LSAB 2 System) with diaminobenzidine chromogenic substrate

(DAKO). Sections were counterstained with hematoxylin solution. Sections were subjectively examined by an investigator who was unaware of the origin of each section evaluated. The presence, intensity, and distribution of target proteins, as determined by immunohistochemical analysis, were subjectively evaluated and scored as mild, moderate, or intense immunoreactivity.

Statistical Analyses

Statistical analyses were performed with a computer software program (Sigma Stat; Statcon). Measurement data were combined for each group, and means ± SEMs were determined. Differences between treatment and control groups were assessed by use of the *t* test or Mann–Whitney rank sum test. Differences among time periods were assessed by use of an ANOVA on ranks. Correlations among variables were analyzed by use of a Spearman rank correlation test. The significance was set at $P \leq 0.05$.

RESULTS

Evaluation of Growth

One rabbit in the ^{153}Sm-EDTMP group died because of complications from anesthesia. No gross abnormalities or morbidity was noted for any remaining rabbits in either group. Rabbits in the ^{153}Sm-EDTMP-treated group had a significant ($P < 0.05$) reduction in total bone growth of the radius compared with control rabbits 8 wk after treatment (Fig. 2). No other significant differences in measurements of the bones or physes were noted.

Histopathologic and Immunohistochemical Evaluations of Physes

For all control rabbits, physeal and articular cartilage had normal histologic morphology and ECM staining for all bones examined. Similarly, for all ^{153}Sm-EDTMP-treated rabbits, articular cartilage had normal histologic morphology and ECM staining for all bones examined. However, 8 (73%) of the 11 rabbits in the ^{153}Sm-EDTMP-treated group exhibited pronounced abnormalities in the histologic morphology of the physeal cartilage in multiple bones (humerus, tibia, and/or radius). The chondrocytes in the zone of proliferation were decreased in number and grouped into clusters. The columnar arrangement of physeal chondrocytes was disrupted and disorganized, clone clusters were noted in various zones of the physis, demarcation between the proliferative and hypertrophic zones was irregular, and retained hypertrophic cells and cartilaginous matrix were present (Fig. 3). Histomorphometric analyses showed a significant ($P < 0.05$) difference in the ratio of physeal cartilage area to the length of the distal radial physis (treatment group, 5.3; control group, 4.2). No significant differences were noted in any other physeal histomorphometric analyses.

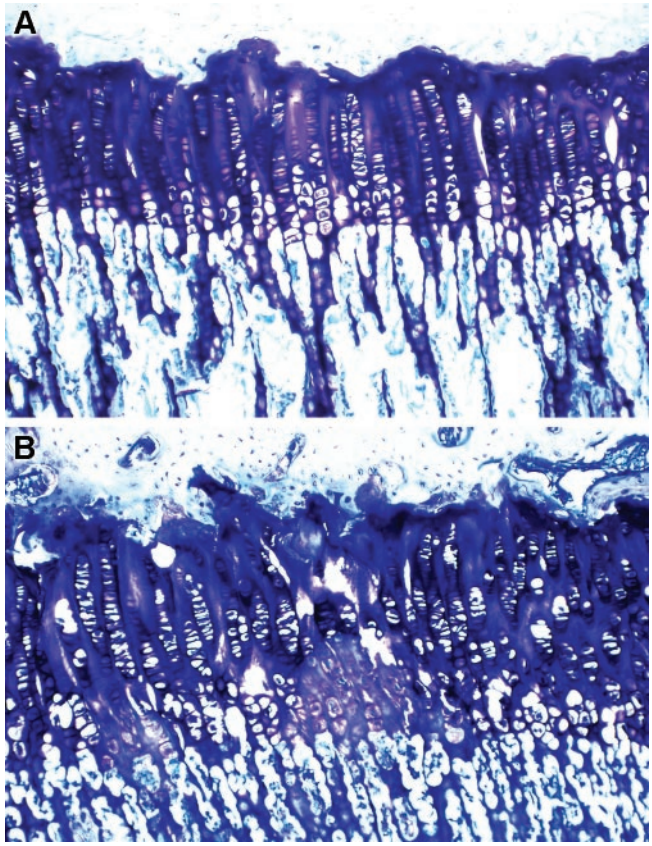

FIGURE 3. Toluidine blue–stained histologic samples of proximal tibial physes from control (A) and treated (B) rabbits. Compared with normal, organized arrangement of chondrocytes in control rabbit, physis of treated rabbit exhibited disrupted and disorganized columnar arrangement as well as irregular lines of demarcation between proliferative and hypertrophic zones; in addition, retained hypertrophic cells and matrix are present in zone of provisional calcification.

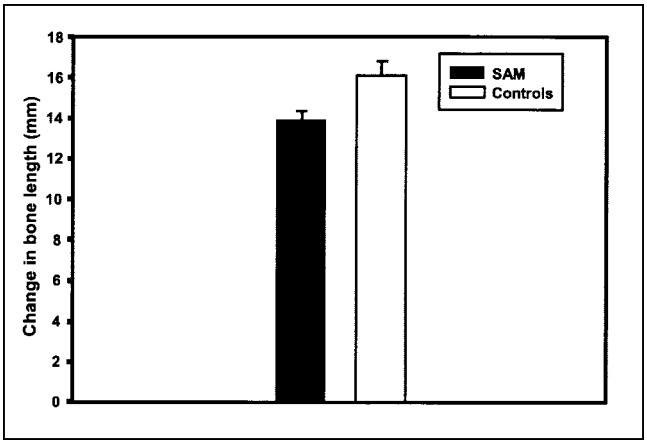

FIGURE 2. Differences in radial bone growth between ^{153}Sm-EDTMP-treated rabbits (SAM) and control rabbits (Controls) 8 wk after injection.

Control rabbits had a significantly ($P < 0.05$) greater degree of positive type X collagen expression than did ^{153}Sm-EDTMP-treated rabbits (Fig. 4). Type X collagen immunoreactivity was exclusively associated with hypertrophic chondrocytes of the physes in both groups. For MMP-13, significantly ($P < 0.05$) more positive expression was seen in control rabbits than in treated rabbits (Fig. 5). MMP-13 expression was primarily seen associated with hypertrophic chondrocytes in both groups. No significant

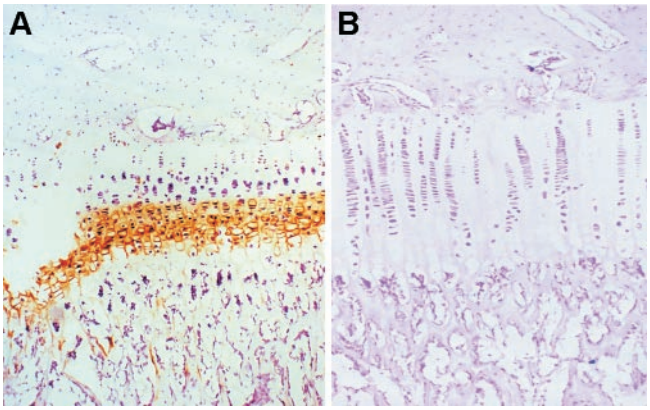

FIGURE 4. Immunoreactivity assessment for type X collagen. (A) Moderate to intense expression (brown stain) of type X collagen was noted in zone of hypertrophy in control rabbit. (B) Minimal expression was noted in treated rabbit.

differences ($P > 0.05$) were noted in MMP-1 expression between groups. However, subjectively, the physeal distribution of MMP-1 expression was diffuse in ^{153}Sm-EDTMP-treated rabbits and was contained within the hypertrophic zone in control rabbits.

DISCUSSION

Differences in radiation sensitivity are attributable to the rate of replication inherent to the critical cells in the treated or irradiated tissues. The Bergonié–Tribondeau law, introduced in 1906, states that the sensitivity of cells to radiation varies directly with their reproductive power and inversely with their degree of differentiation (*17,18*). Ionizing radiation affects all phases of physeal activity, but especially chondrocytes and small blood vessels (*1,4,19*). Chondrocytes in the zone of proliferation in physeal cartilage are rapidly dividing, considered differentiating intermitotic cells, and therefore are radiosensitive (*20*). Cells in the reserve and hypertrophic zones are not dividing; therefore, they are less likely to express radiation damage. In addition, the metaphyseal vasculature is lined with endothelium. Radiation damage to these blood vessels results in the irregular production of osteoid and faulty bone formation (*4*). Damage to proliferating chondrocytes leads to a disruption of the anisotropic environment of the cartilage (*1*), and metaphyseal vascular injury results in deficient absorptive processes, each contributing to growth disturbances. Histopathologically, these features were consistently seen in the present study as haphazardly arranged chondrocyte columns and irregular lines of demarcation between the zones of proliferation and hypertrophy in treated rabbits. Retained hypertrophic cells were apparent in the zone of mineralization, likely as a result of damage to the metaphyseal vasculature and the failure of the chondrocytes to mature normally.

The absolute shortening after irradiation of long bones is a critical detrimental sequela of radiation therapy. Studies evaluating external beam therapy have shown that restoration of the normal columnar arrangement can occur; however, the likelihood and time frame for this process to occur depend on the total dose of radiation (*6,20*). These studies have reported that the dose–effect relationship may be particularly steep at doses of 15–30 Gy, with the saturation dose occurring at levels of 25–40 Gy (*1,17,20*). In a study evaluating a dose–effect relationship as a function of age, it was noted that when the parameter used for estimating the radiation effect was the overall shortening of the irradiated limb, greater effects were seen in less mature bones. However, when growth remaining after irradiation was considered, the age at the time of irradiation did not influence the final effects; the total dose of irradiation was the most important factor (*6*). Limitations of the present study are the lack of quantification of the total ^{153}Sm-EDTMP dose delivered to the physeal cartilage and the evaluation of the cartilage at only one time point after irradiation. However, in a study that quantified the effects of external radiation on growing long bones in rats, growth inhibition could not be produced by less than 5 Gy (*17*). Additional studies have supported the minimal stunting dose and have established saturation levels at which increasing doses do not produce additional effects (*1,20*). In the present study, it is important to recognize that in the interim of 8 wk, physeal cartilage was affected such that histologic evidence of disorganization and radiation damage was consistently present.

Normal closure time for the proximal radial physis in a rabbit is 90–110 d (*21*). The proximal radial physis was the only growth plate that had undergone complete ossification at the time of evaluation. The reduction in radial bone

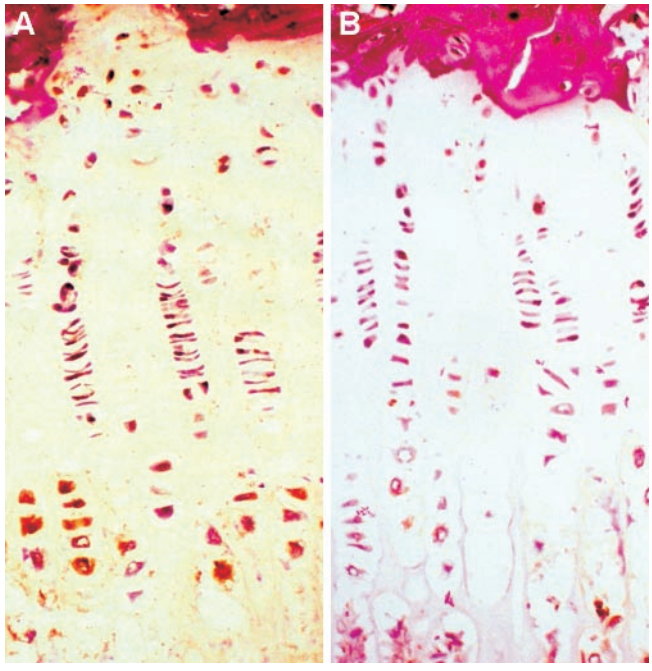

FIGURE 5. Immunoreactivity assessment for MMP-13. (A) Mild to moderate staining (brown stain) in zone of hypertrophy indicated expression of MMP-13 in control rabbit. (B) Treated rabbit exhibited no expression of marker.

growth and histologic abnormalities noted in this study are likely the result of the localization of ^{153}Sm-EDTMP in the zone of provisional calcification, thus exposing the metaphyseal vasculature and proliferating chondrocytes to ionizing radiation damage. The acute insult may have caused prolonged disruption of the orderly columnar organization of the growth plate, and the interval between the administration of radiation and closure time may not have been sufficiently long to allow restoration of the critical cell population because of the overall short growth period of the animal. In addition, whereas the metaphyseal vasculature is radiosensitive, it has been shown to regenerate more quickly than proliferating chondrocytes (*4*). This process would allow the deposition of osteoid to occur, resulting in premature physeal closure and thus in overall shortening of the bone. In all other bones evaluated (humerus and tibia), growth plates remained open until the time of sacrifice, potentially masking any clinical expression of growth impairment.

In the zone of proliferation, cells multiply and secrete an ECM composed primarily of type II collagen (*22*). The cells reach maximum size in the zone of hypertrophy, accompanied by a decrease in the matrix volume and degradation of type II collagen. Type X collagen is synthesized and found solely in the zone of hypertrophy. Calcification ensues, and type X collagen synthesis is arrested (*22–24*). In this study, the evaluation of type X collagen served as a marker for normal endochondral ossification.

MMPs are also important for the development and maintenance of the ECM of cartilage. Under normal physiologic conditions, the synthesis of MMPs is maintained at a level that helps to balance ECM synthesis, whereas under pathologic conditions, the upregulation of MMPs is noted. Recent studies have reported that MMP-13 (collagenase 3) expression is associated with chondrocytes undergoing hypertrophy and that MMP-13 levels in the zone of hypertrophy parallel the expression of type X collagen (*25*,*26*). The degradation of the cartilaginous matrix is necessary to permit proliferating chondrocytes to undergo hypertrophy and progress through the orderly process toward calcification and mineralization. Therefore, increases in MMP-13 expression may indicate that this proteinase is responsible for matrix degradation during endochondral bone formation (*25*), suggesting that MMP-13 plays an integral role in cell enlargement and cartilage calcification (*25*,*26*). In the present study, the localization of ^{153}Sm-EDTMP in the metaphysis caused irradiation of the critical cell population, the proliferating chondrocytes, thus disrupting the normal growth cycle of the physeal cartilage. It is likely that the degradation of the ECM did not occur normally, thereby disrupting normal endochondral ossification. The lack of expression of type X collagen and MMP-13 in the treatment group indicated a failure of chondrocytes to mature normally after irradiation with ^{153}Sm-EDTMP.

These data suggest that clinically significant damage to the developing physeal cartilage may occur as a result of the intravenous administration of ^{153}Sm-EDTMP at the dose studied. In these juvenile rabbits, proliferating chondrocytes were exposed to ionizing radiation emitted from ^{153}Sm-EDTMP localized in the periphyseal bone. This exposure resulted in significant alterations in bone length, physeal area and morphology, and the synthesis of physeal chondrocytes. An expansion of this study will evaluate the effects of different doses and the overall effect of each dose on bone length at maturity. It is encouraging that articular cartilage damage, gross limb abnormalities, and associated morbidity were not noted in any of the rabbits in this study. However, the effects on radial bone growth and the consistent and widespread disturbances in the developing physeal tissues may have immediate clinical implications for the use of ^{153}Sm-EDTMP in the treatment of juvenile osteosarcoma. Further investigations regarding the effects of radiopharmaceuticals on developing physeal cartilage are warranted.

CONCLUSION

Histologic derangement of growing physes occurred after ^{153}Sm-EDTMP injection at the dose studied. Clinically significant damage to immature physeal cartilage may occur and may have immediate clinical implications for the use of ^{153}Sm-EDTMP in juvenile patients; however, it is unclear at this time whether the results obtained in rabbits can be transferred to juvenile osteosarcoma patients.

ACKNOWLEDGMENTS

This work was funded by the University of Missouri Committee on Research and the Comparative Orthopaedic Laboratory of the University of Missouri. We thank Dr. Derek Fox, Dr. Juri Ota, and Jan Kunkel for their technical assistance.

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