Changes of Grip Strength, Articular Cartilage and Subchondral Bone in Monoiodoacetate-Induced Osteoarthritis in Rats
(Perubahan pada Kekuatan Genggaman, Rawan Artikul dan Tulang Subkondral dalam Osteoartritis Aruhan Monoiodoasetat pada Tikus)

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ABSTRACT
Osteoarthritis is a degenerative disease affecting articular cartilage among the elderly. The intra-articular moniodioacetate injection is one of the most widely used methods to induce osteoarthritis in animals. While the effects of moniodioacetate on cartilage are well-characterized, its effects on subchondral bone remodeling are less studied. The purpose of this study was to determine the changes of the grip strength, articular cartilage structure and subchondral bone remodeling in moniodioacetate-induced osteoarthritis in rats. Three-month-old male Wistar rats were assigned to normal control (n=6) and osteoarthritis group (n=6), which received intra-articular injection of 4 mg/50 µL moniodioacetate solution once at the left knee of hindlimb. The rats were monitored for four weeks. The grip strength test was performed before injection and every week after injection. After four weeks, the femurs with intact cartilage were harvested for histomorphological analysis. Grip strength was reduced significantly in the osteoarthritic rats compared to the normal rats (p<0.05). Food intake was reduced significantly one week following moniodoacetate-induction (p<0.05), but it stabilized afterwards. Mnoiodoacetate injection increased cartilage erosion and osteoclast number in the subchondral bone of the osteoarthritic rats compared to the normal rats (p<0.05). However, it did not affect body weight, subchondral bone osteoblast activity, mineralization and microstructure of osteoarthritic rats (p>0.05). As a conclusion, moniodioacetate-induced osteoarthritis affects the cartilage and increases osteoclast formation in the subchondral bone of rats.

Keywords: Femur; moniodioacetate; osteoarthritis; subchondral bone

ABSTRAK
Osteoartritis ialah penyakit degeneratif yang merosakkan rawan artikul dalam kalangan warga tua. Suntikan intra-artikul moniodioasetat merupakan salah satu kaedah yang paling biasa digunakan untuk mengaruh osteoartritis pada haiwan. Walaupun kekuatan moniodioasetat pada rawan telah diperincikan, kekuatannya terhadap penukargantian tulang subkondral kurang dikaji. Tujuan kajian ini adalah untuk menentukan perubahan kekuatan genggaman, struktur rawan artikul dan penukargantian tulang subkondral dalam osteoartritis aruhan moniodioasetat pada tikus. Tikus Wistar berumur tiga bulan telah dibahagi kepada kumpulan kawalan normal (n=6) dan osteoartritis (n=6) yang menerima suntikan intra-artikul larutan moniodioasetat pada 4 mg/50 µL sekali pada sendi kiri kaki belakang. Tikus tersebut telah diperhatikan selama empat minggu. Ujian kekuatan genggaman telah dilakukan sebelum suntikan dan setiap minggu selepas suntikan. Selepas empat minggu, suntikan moniodioasetat pada rawan telah diadakan untuk analisis histomorfometri. Kekuatan genggaman telah menunjukkan secara signifikan pada tikus yang mempunyai osteoartritis berbanding dengan tikus normal (p<0.05). Pengambilan makanan telah berkurang secara signifikan satu minggu selepas aruhan moniodioasetat (p<0.05), tetapi ia menjadi stabil selepas itu. Suntikan moniodioasetat telah meningkatkan hakisan rawan dan bilangan osteoklas dalam tulang subkondral pada tikus yang mempunyai osteoartritis berbanding dengan tikus normal (p<0.05). Walau bagaimanapun, ia tidak mengganggu berat badan, aktiviti osteoblas, mineralisasi dan mikrostruktur pada tulang subkondral tikus osteoartritis (p>0.05). Secara kesimpulannya, osteoartritis aruhan moniodioasetat memberi kesan terhadap rawan dan meningkatkan pembentukan osteoklas dalam tulang subkondral tikus.

Kata kunci: Femur; moniodioasetat; osteoartritis; tulang subkondral
INTRODUCTION

Osteoarthritis (OA) is a degenerative disease affecting movable joints of the body. The deterioration involves the entire joint, including the articular cartilage, synovium and the subchondral bone (Litwic et al. 2013; Sinusas 2012). OA is characterised by motion-triggered pain, swelling, and joint stiffness, limiting its range of motion (Sinusas 2012). OA progresses slowly over time, eventually leading to joint failure (Litwic et al. 2013). According to the Global Burden of Disease 2019 Study, 528 million people worldwide suffer from knee OA. OA is the 15th top contributor of years lived with disability, and the top 37th top contributor of disability-adjusted life years (Global Burden of Disease Collaborative Network 2020).

The risk factors of osteoarthritis include trauma, mechanical forces, inflammation and metabolic derangements (Ayhan et al. 2014). The role of inflammation in the pathogenesis of OA is not clearly understood, although it has been linked to OA. Questions remain whether inflammation triggers OA or is secondary to OA (Ayhan et al. 2014). Some studies reported that OA is characterised by low-grade chronic inflammation involving innate immunity. Infiltration of inflammatory cells into the synovium can be noticed in the early stages of OA but is more prominent in the advanced stage (Robinson et al. 2016). The presence of multiple inflammatory mediators in the synovial fluid induces the release of matrix metalloproteinases and other hydrolytic enzymes, culminating in cartilage degradation due to the dissolution of proteoglycan and collagen (Robinson et al. 2016).

Articular cartilage is the most affected joint tissue affected by OA. Owing to the lack of vasculature and innervation, the cartilage itself is not the source of inflammation or discomfort, at least at the early stages of the disease. The cause of pain arises primarily from the joint capsule, synovium, subchondral bone, ligaments, and peri-articular muscles (Sharma et al. 2017). As the disease progresses, further alterations in tissues surrounding the joints are affected, resulting in altered bone remodelling, osteophyte development, peri-articular muscle weakness, ligament laxity, and synovial effusion (Dulay et al. 2015). There is currently no cure for OA. Analgesics are the most commonly prescribed medications to alleviate the pain associated with OA (Hunter 2011).

Experimental models of OA include spontaneous, surgical and chemical models. Chemically induced models are more replicable than spontaneous models and require fewer invasive procedures than surgical models, making them easier to be implemented and allowing researchers to study OA lesions at various stages (Pitcher et al. 2016). Monoiodoacetate (MIA) is the most commonly used agent in chemically induced OA models because it closely resembles the histological and pain-related behaviour of human OA. Thus, the MIA-induced OA model is more predictive of pain-alleviating drug efficacy (Samvelyan et al. 2021). Furthermore, MIA causes chondrocyte cell death, which leads to cartilage degeneration and subchondral bone changes (Pitcher et al. 2016). Though OA has long been regarded to be a primary articular cartilage condition, the role of subchondral bone in OA physiopathology is becoming more recognized (Suri & Walsh 2012). Subchondral bone sclerosis, along with increasing cartilage degradation, is usually regarded as a characteristic of OA (Burr & Gallant 2012; Henrotin et al. 2012). Subchondral bone is hypomineralized and of low quality regardless of the increased trabeculae number and bone volume due to excessively high local bone turnover with several studies reporting microdamage, edema-like lesions, and bone cysts in subchondral bone (Li et al. 2013; McErlain et al. 2012). Despite mounting evidence that subchondral bone plays a role in the pathogenesis of OA, changes in subchondral bone structure and remodeling in the MIA model are not well characterized. This study aims to determine the structural and remodeling changes of subchondral bone in a rat OA model induced by MIA.

MATERIALS AND METHODS

MATERIALS

MIA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in normal saline (4 mg in 50 µL) prior to administration.

ANIMALS

Twelve male Wistar rats (250-300 g) were maintained at the Animal Laboratory of the Pharmacology Department, UKM (Cheras, Malaysia) under a standard temperature of 25 ± 3 °C and 12 h:12 h light-dark cycle. Rats received standard rat chow and tap water ad libitum throughout the experiment. After two weeks of acclimatization, the rats were divided into two groups (n = 6 for each group), namely normal control (non-OA rats) and OA group (injected with 4 mg/50 µL MIA intra-articularly once at the articular space of the left knee (hindlimb) at the beginning of the study (day 0)). MIA
at this dose was reported to induce OA effectively in rats (Al-Saadi et al. 2021). Four weeks after MIA induction, the rats were euthanized with an overdose of ketamine/xylazine/zoletil injection. The experimental period was commonly used in drug screening based on this model, and it results in significant changes in pain behaviours, histology, and radiology findings (Otis et al. 2017). Five days and two days before euthanization, the rats were injected subcutaneously with 10 mg/mL of calcein to label the bone for dynamic parameters. The left femurs were harvested and stored in neutral buffered formalin at room temperature. This study was performed following the guidelines and ethics of laboratory animals set by Universiti Kebangsaan Malaysia. The study procedures were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (approval code: FAR/PP/2018/KOK YONG/26-SEPT./946-JAN.-2019-DEC.-2020).

**GRIP STRENGTH TEST**

Grip strength was tested on twelve rats using modified Kondziela’s inverted screen test (Deacon 2013). The rats were placed at the centre of a wire mesh screen. The clock was started within 2 s after the screen was inverted (with the head of the rats declining first). The screen was held steadily 60 cm above a padded surface. The time the rats released their hindlimb and the time they fell were recorded. The test was performed before MIA injection and weekly until the rats were euthanized.

**HISTOLOGY OF THE DISTAL FEMUR CARTILAGE AND SUBCONDRAL BONE**

The histomorphometric examination was performed using decalcified and undecalciﬁed femurs. The left femur was cleaned of soft tissue and sawed into two halves with the cartilage still intact. Half of the femur was decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 30 days and embedded in parafﬁn wax. Histological sections (5 μm) were stained with safranin O and fast green stain and scored by a blinded anatomist using the modiﬁed Mankin scoring (MMS) system. The maximum score obtainable using the MMS system was 15 (Salo et al. 2002). The grading system for each aspect is summarised in Table 1. Slides were also stained with haematoxylin and eosin and evaluated for subchondral bone cellular parameters.

The undecalciﬁed half of the femur was embedded in polymethyl methacrylate (Polysciences, PA, USA). The unstained sections (8 μm) were analyzed for subchondral bone dynamic parameters. In addition, the sections were stained with von Kossa method and analyzed for structural bone indices. Weibel Grid technique was used to obtain cellular and dynamic parameters at the subchondral bone of the rats.

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**TABLE 1. Aspects of modified Mankin scoring system**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Cellularity</th>
<th>Matrix staining</th>
<th>Tidemark integrity</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth surface/normal</td>
<td>Normal arrangement</td>
<td>Normal staining</td>
<td>Normal and intact</td>
<td>0</td>
</tr>
<tr>
<td>Roughened surface/single crack or area of deamination</td>
<td>Clustering in superficial layer or loss of cells up to 10%</td>
<td>Slight loss of stain</td>
<td>Disrupted</td>
<td>1</td>
</tr>
<tr>
<td>Multiple cracks/moderate delamination</td>
<td>Disorganisation or loss up to 25%</td>
<td>Moderate loss of stain</td>
<td>X</td>
<td>2</td>
</tr>
<tr>
<td>Fragmentation in cartilage or severe delamination</td>
<td>Cell rows absent or loss up to 50%</td>
<td>Severe loss of stain</td>
<td>X</td>
<td>3</td>
</tr>
<tr>
<td>Loss of fragments</td>
<td>Very few cells present X</td>
<td>No stain present</td>
<td>X</td>
<td>4</td>
</tr>
<tr>
<td>Complete erosion to tidemark</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5</td>
</tr>
<tr>
<td>Erosion beyond tidemark</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>6</td>
</tr>
</tbody>
</table>
STATISTICAL ANALYSIS

Based on Shapiro-Wilk test, dynamic and structural histomorphometric parameters were normally distributed and analyzed using independent sample t-test. For static histomorphometric parameters that were not normally distributed, Mann-Whitney U test was used. Comparisons of body weight, food intake and grip strength test adopted a time × treatment design, thus, were interpreted using mixed-design analysis of variance (ANOVA) with simple effect analysis. Data for body weight, food intake, grip strength test and bone histomorphometric parameters were displayed as mean ± standard error of the mean. Histological scores of the joint were presented as median (interquartile range). A p-value < 0.05 was considered as statistically significant. Statistical analysis was conducted using Statistical Package for Social Sciences version 20 (IBM, Armonk, USA).

RESULTS

Body weight was measured before (week 0) and every week (week 1-4) after MIA induction. There was a significant ‘time’ (p<0.05) and ‘time × treatment’ (p<0.05) effect on body weight. All experimental groups showed a significant increase in body weight after MIA induction every week (p<0.05 vs previous weeks). However, there was no significant difference observed in the body weight between the normal and OA group throughout the study period (p>0.05) (Figure 1).

Food intake was measured weekly after MIA induction. There were significant ‘time’ (p<0.05) and ‘time × treatment’ (p<0.05) effects on food intake. At week 1, food intake in the OA group decreased significantly compared to the normal group (p<0.05). Food intake in the normal group increased significantly every week compared to week 0 (p<0.05). In the OA group, a significant increase in food intake was observed at week 4 compared to week 2 and 3 (Figure 2).
Grip strength was measured before (week 0) and every week (week 1-4) after MIA induction. There were significant ‘time’ (p<0.05) and ‘time × treatment’ (p<0.05) effects on grip strength. The time taken for the OA rats to release their left hindlimb from the wire mesh screen was reduced significantly at week 3 and 4 compared to the normal control (p<0.05). The time taken for normal rats to release their left hindlimb increased significantly at week 1 compared to week 0 and at week 4 compared to week 0 and 2 (p<0.05). The time taken for OA rats to fall from the wire mesh increased significantly compared to the normal group at week 2 (p<0.05). The time taken for OA rats to fall from wire mesh also increased significantly at week 2 compared to week 0 and 1 (p<0.05) (Figure 3).

Safranin O-stained micrographs from the femur showed thinning of articular cartilage (AC) in the OA rats compared to the normal rats (Figure 4). Significantly increased modified Mankin score were observed in structure (p = 0.002), cellularity (p = 0.002), matrix staining (p = 0.002), tidemark integrity (p = 0.001) and total score (p = 0.002) in the OA rats compared to the normal rats (Figure 4).

The static histomorphometric indices for subchondral bone included osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), eroded surface (ES/BS), osteoid surface (OS/BS) and osteoid volume (OV/BV). Results showed that Oc.S/BS was significantly increased in the OA rats (p = 0.004) compared to the normal rats. However, there was no significant difference observed in Ob.S/BS, ES/BS OS/BS and OV/BV of osteoarthritic rats compared to normal rats (Figure 5).

The dynamic histomorphometric indices for subchondral bone included single-labelled surface (sLS/BS), doubled-labelled surface (dLS/BS), mineralising surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR). The results showed no significant difference in sLS/BS, dLS/BS, MS/BS, MAR and BFR of the osteoarthritic rats compared to normal rats (Figure 6).

The structural histomorphometric indices for subchondral bone included bone volume/total volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), and trabecular bone separation (Tb.Sp). Results showed no significant difference in BV/TV, Tb.Th, Tb.N and Tb.Sp in the osteoarthritic rats compared to normal rats (Figure 7).
FIGURE 3. Comparison of grip strength test between groups across study period; time taken for rats to release hind limb (A); time taken to drop down (B). A gradual decrease of the time in releasing hind limb is observed in rats with osteoarthritis from week 2 onwards but this is not observed for the total drop down time. Values are expressed as mean ± standard error mean (n = 6). Statistical significance is evaluated by mixed-design ANOVA. *p < 0.05 vs normal control at same time point; †p < 0.05 vs week 0, ‡p < 0.05 vs week 1, ‡‡p < 0.05 vs week 2 of the same group.
FIGURE 4. Micrograph of safranin O-stained knee sections for left femur of normal (20× magnification) (A) and osteoarthritic (B) rats; Knee histology score using modified Mankin score system for structure (C), cellularity (D), matrix staining (E), tidemark (F) and total histology score (G). Rats administered with monosodium iodoacetate show degenerative cartilage changes as evidenced from the micrograph and increased Mankin scores. Values are expressed as median and interquartile range. Statistical significance is evaluated by Mann-Whitney U test. *p < 0.05 vs normal control. The symbol ‘x’ in the box plot represents the mean value while the horizontal line in the box represents the median of the group.
FIGURE 5. Micrograph of H & E-stained knee sections for left femur of normal (40× magnification) (A) and osteoarthritic (B) rats; Static histomorphometric indices of subchondral bone for Ob.S/BS (C), Oc.S/BS (D), ES/BS (E), OS/BS (F) and OV/BV (G). Oc.S/BS increases significantly in the rats with osteoarthritis compared to normal rats. Values are expressed as mean ± standard error mean (n = 6). Statistical significance is evaluated by independent samples t-test. *p < 0.05 vs normal control.
FIGURE 6. Micrograph of calcein-labelled knee sections for left femur of normal (20× magnification) (A) and osteoarthritic (B) rats; Dynamic histomorphometric indices of subchondral bone for sLS/BS (C), dLS/BS (D), MS/BS (E), MAR (F) and BFR (G). There are no differences in the bone dynamic histomorphometric indices between rats with and without osteoarthritis. Values are expressed as mean ± standard error mean (n = 6). Statistical significance is evaluated by independent samples t-test.
DISCUSSION

This study examined the grip strength, articular cartilage and subchondral bone changes associated with MIA-induced OA in male Wistar rats. No significant changes in body weight were observed between the two groups throughout the study. Food intake of the OA group was lower than the normal rats during the first week after induction, but no significant difference was observed from week 2-4. Grip strength was reduced and articular cartilage degradation was increased in the OA group compared to the normal healthy group. MIA induction significantly increased Oc.S/BS, but there were no
significant differences in Ob.S/BS, ES/BS, OS/BS and OV/BV between the two groups. MIA-induced OA did not affect the structural indices and dynamic indices of subchondral bone in the OA rats compared to the normal control.

OA is associated with joint pain that increases with motion (Sinunas 2012). In some experiments, joint pain due to OA could reduce body weight gain and exploratory activity, especially vertical activity (rearing) of the rats (Cobos & Portillo-Salido 2013). In our study, MIA-induced OA did not affect the body weight of rats. Although a significantly lower food intake was observed in the OA group than the normal group one week after MIA induction, the difference diminished after 2 weeks. It is postulated that the joint pain could affect the vertical rearing of the OA group, leading to reduced food intake. After the first week, the rats might have adapted to using the unaffected hindlimb to reach for food, resulting in normal food intake. The grip strength of the OA group was reduced, particularly at the hindlimb, 3 weeks after MIA induction, indicative of pain and progressive deterioration of the joint tissue. The let-go time of the left hindlimb is a more sensitive measure of grip strength in this study because OA was induced at the hindlimb of the rats. The rats also tended to grip with their forelimbs before falling, thus prolonging their overall falling time.

MIA inhibits the aerobic glycolysis pathway of the cells. Intra-articular injection of MIA inhibits glyceraldehyde-3-phosphatase dehydrogenase in chondrocytes, resulting in chondrocyte death and cartilage degradation. The cellular and cartilage debris cause infiltration of immune cells, synovitis and inflammation (Steinmeyer et al. 2018). The modified Mankin scoring system (Salo et al. 2002) is used to evaluate the extent of cartilage damage in this study. The thinning of cartilage and increased Mankin scores in OA rats parallels with the findings of various OA models induced by papain (Murat et al. 2007), MIA (Asjed et al. 2019), type II collagenase (Henson & Vincent 2008), single impact load (Yang et al. 2020) and anterior cruciate ligament transection (ACLT) (Yang et al. 2020).

The altered mechanical loading on the thinning cartilage changes the subchondral bone, which serves as structural support and shock absorber, reducing around 30% of the loading exerted to the joint (Ji et al. 2018). During the early stages of OA, the subchondral bone plate becomes thinner and more porous as the cartilage degrades. With increased trabecular separation and decreasing trabecular thickness, subchondral trabeculae degenerate. In late OA, the subchondral bone plate and trabeculae thicken, accompanied by subchondral bone sclerosis and decreased bone marrow spacing (Hügle & Geurts 2016). This study investigated the effects of MIA-induced OA on subchondral bone cellular, dynamic and structural histomorphometry parameters after 4 weeks.

Cellular bone histomorphometry provides a snapshot of bone cell distribution and their activity in vivo, estimated from the amount of osteoid and extent of resorption cavities (Kulak & Dempster 2010). OcS/BS was significantly higher in the OA group in this study, indicating increased osteoclast formation. In a study by Guzman et al. (2003), increased osteoclasts activity was observed on day 7 at the junction of damaged necrotic cartilage and subchondral bone in MIA-induced osteoarthritic rats. Chin et al. (2019) reported increased ES/BS and OcS/BS in rats with OA after 4 weeks of induction. Their findings pointed to an increase in bone resorption activity due to OA, similar to the current study.

Dynamic bone histomorphometry assesses bone mineralisation activities in vivo (Kulak & Dempster 2010). According to Bagi et al. (2015), active remodelling at the subchondral bone was observed after 10 weeks in rats with medial meniscectomy. Similarly, sLS/BS, dLS/BS, MS/BS, MAR and BFR were significantly decreased in rats with ACLT-induced OA after 20 weeks compared to normal rats, which signifies a decrease in bone formation rate (Namhong et al. 2020). In this study, no significant changes were observed in dynamic parameters between the normal and OA group, suggesting that OA did not affect the mineralization activities of subchondral bone at this stage. This observation could be due to the duration used of the current study, which reflects the early changes of OA (osteo lytic) rather than late changes (anabolic).

The microstructure of subchondral bone was estimated determined by structural histomorphometric parameters in this study (Kulak & Dempster 2010). No significant difference was observed in the structural parameters between the normal and the OA group, suggesting that MIA-induced OA did not affect the subchondral structure of the rats at this stage. In contrast, Zhang et al. (2011) reported a significant decrease in BV/TV and Tb.Th with a significant increase in Tb.Sp in rabbits with ACLT-induced OA after 60 days. Due to the short duration of the current study, it is postulated that the osteoclastic changes observed had not impacted the microstructure of the subchondral bone.
Considering all results together, MIA induction might have significantly deteriorated articular cartilage after one week, reducing the food intake of rats by impairing their hindlimb function. Reduced grip strength in rats at the third and fourth week post induction was another sign of progressive OA damage. Furthermore, the cartilage deterioration might have altered mechanical loading to the subchondral region, driving osteoclast formation to absorb the damaged bone. However, the mineralization and structure of the subchondral bone were not affected significantly, probably because the changes in bone remodeling are still early. These observations imply that a 4-week MIA induction in rats mimics the early stage of OA.

There are some limitations to this study. The femur alone was chosen for histological analysis. OA is now considered a whole-joint disease that affects all tissue surrounding the joint apart from articular cartilage and subchondral bone (Gallo et al. 2017). These tissues include the meniscus, ligaments, synovial membrane and infrapatellar fat pad (Lampropoulou-Adamidou et al. 2014), which should be examined in the future. Circulating biochemical markers, such as cartilage degradation markers, bone remodelling markers and inflammatory markers, could be included to consolidate histology findings. Micro-computed tomography is the best instrument for evaluating subchondral bone microarchitecture, but it was not available to the researchers at the time of the study. Identification of cell types was based solely on bone morphology without immunostaining, like tartrate-resistant acid phosphatase for osteoclasts. Female rats should be considered in future studies because OA is severe in women, and its prevalence and incidence increase after menopause, probably due to oestrogen loss during menopause (Sur & Chakravorty 2016). Nevertheless, this study provides a picture of subchondral bone remodeling in the MIA-induced rat model used widely in OA research.

**CONCLUSION**

MIA-induced OA increases cartilage degradation, osteoclast number of subchondral bone and reduces grip strength of the rats after 4 weeks. This model could be used to evaluate the changes of cartilage and subchondral bone remodeling in OA, and the protective effects of pharmacological candidates on both joint components. Additional parameters such as biochemical parameters and immunostaining could be added in the future to consolidate the findings of this study.

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