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Effect of Recombinant Human Growth Hormone (rhGH) on the Expression of NHE1 and AE2 Membrane Transporters on Ex Vivo Bone Growth in a Rat Model

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ABSTRACT

Longitudinal growth of bones occurs through endochondral ossification within the epiphyseal growth plate (GP) of the skeleton. The effect of growth hormone (GH) on the expression of the membrane sodium hydrogen exchanger (NHE1) and anion exchanger (AE2) during skeletal growth was investigated using an ex-vivo model of rat bones. Tibia and metatarsal bones from day 10 pups were used for the investigation. The bones showed a steady rate of growth in the DMSO control media after 48 hours of incubation. The addition of recombinant human growth hormone (rhGH) to the culture media resulted in a direct stimulation of the whole bone growth, the whole growth plate length, and the whole growth plate density of chondrocytes. No significant changes (P>0.05) between the DMSO control and rhGH-treated were noted. Incorporation of membrane inhibitors (NHE1 and AE2) (5-(N-ethyl-N-isoprophyl) amiloride [EIPA] and (4,4-diiodothiocyano-2,2-stilbenedisulphonate) [DIDS] respectively in the culture media in the presence of rhGH remarkably suppressed the whole bone growth, whole GP length, GP chondrocytes population, and tissue expression of NHE1 (Na^+/H^+ antiporter) and AE2 (HCO_3^- anion exchanger) along the GP. Using NHE1 and AE2 rabbit polyclonal antibodies, tissue expression of Na^+/H^+ antiporter and AE2 HCO_3^- anion exchangers were significantly higher in rhGH than DMSO control cultured bones. The hormonal treatment appeared not to have direct stimulating effects on the growth of the bone, but may have an indirect metabolic effect that enhances chondrocyte proliferation and differentiation. EIPA and DIDS Plasma membrane inhibitors can still suppress longitudinal bone growth in the presence of rhGH.

Keywords: Long bone growth, Membrane transporters, Growth plate chondrocytes, Growth hormone.

INTRODUCTION

During mammalian skeletal bone development, the long bones increase in length by means of endochondral ossification (Phornphutkul & Gruppuso, 2009; Abubakar et al., 2016). The growth of the long bones is primarily achieved through chondrogenesis in the proliferative and hypertrophic zones of the growth plate (GP) region of the long bone (Staines et al., 2014; Sun and Beier, 2014). The longitudinal growth of bone is orderly controlled by systemic, local paracrine and mechanical factors (Forriol et al., 2010; Abubakar et al., 2019; Etschmaier et al., 2024). It involves the coordinated transition of cartilage to bone through endochondral bone formation within the GP of the long bone (Staines et al., 2014; Abubakar et al., 2022; Oichi et al., 2023).

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Histologically, the GP is a thin layer of cartilage located between the epiphyseal and metaphyseal proximal and distal regions of the long bones (Villavicencio-Lorini et al., 2010, Abubakar et al., 2022), it is divided into the reserve (pre-chondrocytes), proliferative, early hypertrophic, and late hypertrophic zones (Villavicencio-Lorini et al., 1994; Lim and Lee, 2023). The reserve zone contains resting chondrocytes, which are also known as mesenchymal stem cells or chondro-progenitor cells (Abubakar et al., 2019; Etschmaier et al., 2024), the resting chondrocytes consist of undifferentiated stem cells that are randomly arranged and have low rates of proliferation. The proliferating capacity of the cells in the zone determines the epiphyseal GP fusion and subsequent cessation of growth in most mammals except rodents (Mackie et al., 2010; Cramer et al., 2021). The proliferative chondrocytes are flattened, oblate in shape and arranged into longitudinal columns. The orientation of the chondrocyte arrangement is directed by the cells at the resting zone (Goldring, 2012; Lim and Lee, 2023). The hypertrophic chondrocytes play an important role within the GP in terms of gross bone lengthening, and it was reported that they produce signaling molecules such as cytokines and growth factors during endochondral ossification (Hojo et al. 2010; Sun and Beier, 2014; Abubakar et al., 2022; Guasto and Cormier-Daire, 2022). The differentiation process of proliferative chondrocytes to hypertrophic chondrocytes is tightly controlled by numerous physiological factors, which are primarily hormonal (IGFs, thyroxine, parathyroid and FGFs) and there are considerable other trace biological molecules (Staines et al., 2014; Sun and Beier, 2014; Beier and Loeser, 2010).

The proliferative chondrocytes undergo critical morphological changes as they gradually transform into hypertrophic chondrocytes, as it has been reported that the transition to the hypertrophic chondrocyte is the most important determinant of the growth of long bones (Emons et al., 2009; Abubakar et al., 2022). Previous studies (Abubakar et al., 2022; Bush et al., 2010; Loqman et al., 2010) have shown that plasma membrane transporter proteins (NKCC1, NHE1 and AE2) were involved in chondrocyte hypertrophy via a regulatory volume mechanism, which contributes to the increase in height. Therefore, this study is an extension of previous works (Loqman et al., 2013; Abubakar et al., 2022) targeting to explore the role of growth hormones along with the two plasma membrane transporters NHE1 (Na^+/H^+) and AE2 (HCO_3^-) on ex vivo long bone growth using metatarsal and tibial bone of rats.

Growth hormone (GH) is well-known physiological molecule that plays a key role in mammalian tissue growth and development (Nyberg and Hallberg, 2023; Luo et al., 2024). GH is a complex, single-chain peptide containing an estimated 191 amino acids with a molecular weight of 22,124 daltons. GH generally stimulates mammalian growth and development, and it has cell proliferative and regenerative capabilities in all tissues (Ranke and Wit, 2018; Yao et al., 2021; Nyberg and Hallberg, 2023). GH is produced by somatotropin cells within the anterior pituitary gland, as reported by Ranabir and Reetu (2011). GH has been reported to contain an anabolic agent that increases serum levels of glucose and free fatty acids in the mammalian system. GH helps in stimulating the release of IGF1 (Ranke and Wit, 2018). The anabolic effect of GH on mammalian tissue is achieved through series of cellular reactions with identified receptors on the targeted cell surfaces (Binder et al., 2011). Increase in height during mammalian growth is the commonly known effect of GH. Carter-Su et al. (2015) reported that stimulation of growth in height by GH may occur through either one of the two proposed mechanisms; by GH binding to receptors on target cells which bring about activation of the MAPK/ERK pathway that can directly stimulate division and multiplication of chondrocytes. Alternatively, the GH effect may be stimulated via the JAK-STAT signaling pathway, which produces insulin-like growth factor 1 (IGF1) from the liver, subsequently stimulating the activity of osteoblasts and chondrocyte to promote bone growth (Lindsey and Mohan, 2016). The serum level of growth hormone increases during early childhood, reaches optimal production at puberty, and then declines; this corresponds to the period when epiphyseal growth plates fuse and longitudinal bone growth ceases, before it starts to decline. During this period, GH helps in the growth and development of bone and cartilage (Tritos and Klibanski, 2016; Zhao et al., 2025).

The current study investigated the role of growth hormone on the physiological effect of two specific plasma membrane transporters (NHE1 and AE2) on the ex-vivo long bones, using metatarsal and tibial bones from rats. We, hypothesized that the linear growth of bone in the presence of growth hormone would not be affected by plasma membrane inhibitors. To test this, metatarsal bone rudiments and tibial bones from 10-day-old post-natal rat pups (P10) were incubated ex vivo for 48 hours in standard culture media incorporated with recombinant human growth hormone (500ngmL^{-1}) either with or without specific plasma membrane inhibitors (NHE1 antiporter and AE2 anion exchanger) using (5-(N-ethyl-N-isoprophyl) amiloride [EIPA] and (4,4-diiodothiocyano-2,2-stilbenedisulphonate) [DIDS] respectively. To achieve this, an attempt was made to determine changes in whole bone length, histologic total growth plate length, total growth plate and hypertrophic zone chondrocytes densities, and immunoperoxidase and fluorescence immunohistochemistry to determine chondrocytes associated expression of Na^+/H^+ and HCO_3^- transporters along the chondrocyte membrane using NHE1 and AE2 rabbit polyclonal primary antibodies.

MATERIALS & METHODS

Animal Preparation

Twenty female SD rat pups 10 days old (P10) were utilized for this investigation. The rats were sourced from the animal resource facilities (ARF, UPM). The choice of age was based on our published work (Abubakar et al., 2019). These young rats were divided into 4 experimental groups without bias. The recombinant growth hormone (HumanKine™, Sigma-Aldrich, USA) alone (rhGH) treated group, (2) rhGH + EIPA, (3) rhGH + DIDS, and (4) DMSO (as vehicle) for plasma membrane inhibitors, details of the experimental grouping are reflected in Table 1.

Table 1: Rats grouping and experimental plan. The choice of dosage for pharmacological inhibitors (EIPA and DIDS) was based on our previous study (Abubakar et al., 2022). While, the dose of rhGH was selected based on the graded preliminary study conducted and dose range reported in literature

Experimental groups	Pharmacological agents used	No of rat pups used	Dosage pharmacological agents used
rhGH alone (test group)	Recombinant human growth hormone	n=5	500ngmL^{-1}
rhGH + EIPA (test group)	Plasma membrane inhibitor (5-(N-ethyl-N-isoprophyl) amiloride [EIPA] against NHE1	n=5	444μM$_+$500ngmL^{-1}
rhGH + DIDS (test group)	Plasma membrane inhibitor (4,4-diiodothiocyano-2,2-stilbenedisulphonate) [DIDS] against AE2	n=5	250μM$_+$500ngmL^{-1}
DMSO vehicle (control)	Dimethyle sulfate used for constitution of EIPA and DIDS	n=5	1mL volume

The rats were humanely killed using an overdose of IP injection of barbiturate (Dolethal®; Vetoquinol, France) at 90mgkg^{-1} as according to the protocol described by Abubakar et al. (2022). The tibial bones along with the middle metatarsal rudiments from each limb were carefully removed alongside the distal and proximal growth plate articular cartilages, using a dissecting stereo-microscope (Huvitz; HSZ-645TR, Korea). To maintain tissue viability, the bones were immediately stored in a medium containing (PBS, α-MEM; 7.5% v/v and BSA; 1mM) at pH 7.4 before further treatment and incubation. The PBS, α-MEM and BSA were all products of Naclai Fesque Inc., Tokyo, Japan.

Tibia and Metatarsal Length Measurements

The initial bone length before incubation was measured at baseline and subsequently at 48 hours after ex vivo incubation. The measurements of the bones were performed using the VIS plus ver.3.50 image analysis and measurement software, Canada. The computer software was connected to the stereo light microscope for tissue dissection. The microscope was fitted with a camera (VIS imaging; UC3010; Malaysia). The microscope and PC were connected via a universal serial bus port (Fig. 1). All bone length measurements were recorded in centimeters (cm) at x6.5 objective magnification of the stereo microscope. The acquired images were analyzed based on standard protocol by Abubakar et al. (2019). The percentage growth rate of the bone was computed by determining the growth differences of the bones after ex vivo bone growth for 48 hours.

The harvested tibia bones were cultured separately inside 6 wells cell incubation plates (flat bottom cell culture plates with lids; Sigma-Aldrich, USA). Metatarsal bone rudiments were incubated inside the 24 well cell culture plates (Nuclon™, delta surface, China). All bone incubations were made within 48-hour period in standard culture medium described earlier at (5% Co_2, 95% air, pH 7.37, and 37°C). Every 24-hour incubation period, the culture media was changed. The bone incubation media used was made up of α-MEM, enriched with sodium glycerol phosphate powder (1mM), L-ascorbic acid powder at 5mgmL^{-1} and penicillin-streptomycin final concentration of 100 IUml^{-1} and 100 μgmL^{-1} (all from Sigma-Aldrich, USA).

Bone Growth Plate (GP) Histology

The bone tissues before and after ex vivo incubation were fixed for 24 hours in a fixative mixture of glutaraldehyde (GA) combined with fresh preparation of ruthenium hexamine trichloride (RHT) (Acros Organics, New Jersey, USA) at pH 7.4 in order to maintain the in-situ shapes of the chondrogenic cells based on the standard protocol by Loqman et al. (2010). Histological processing of the bones was carried out using automatic tissue processor within 12 hours (TP 1020 semi-closed benchtop tissue processor; Leica, Singapore). After processing, the bones were embedded in paraffin wax inside a plastic cassette container, based on the histological protocol described by Abubakar et al., (2022). The bone samples were cut into 5μm longitudinal serial sections using a Reichert-Jung 2045 multicut rotary microtome. After removal of the paraffin wax with xylene solution and reverse hydration with decreasing concentrations of ethanol solution, the thin tissue sections were stained with toluidine blue O (Acros Organics, New Jersey, USA) in PBS (pH 5.6, 30s, 23°C). The tissue sections were finally placed on the cleaned microscope glass slides (Menzel-Glaser, Germany), sections were rinsed in cleaned water and air-dried prior to mounting with the cover slip.

Fig. 1: The stereo dissecting microscope alone PC used for bone length measurement.

Histological Cells Quantification

The distal and proximal growth plates (GPs) images were captured using a fluorescence microscope (Nikon Eclipse Ti-S, Japan) fitted with a 20X [numeric aperture = 0.5WD (82000μm)] dry objective lens. For determination of the entire growth plate length, the unique arrangement of the chondrocyte within the GP was used as the criteria (Abubakar et al., 2019). The GP length and its various regions were carefully demarcated and measured based on an existing standard protocol (Abubakar et al., 2019). The five zones of the GP identified were demarcated with freehand sketching: the resting chondrocyte region, the chondrocyte proliferation zone, early hypertrophic

chondrocytes, late hypertrophic chondrocytes and the mineralized zone at the bottom of the hypertrophic zone.

In each growth plate, the whole GP length and the entire GP chondrocyte population at the five zones were determined and the number of chondrocytes was estimated. The counting of the chondrocytes was carried out with a computer analytical software (NIS-Element BR4.20.00 64-bit, Nikon, Japan). The software was fitted with a Nikon digital sight camera DS-Fi2, K16850. (Fig. 2). The images of the GP acquired were arranged to make the orientation of the GP upright on the screen of the computer prior to image acquisition in each case. The number of the chondrocytes was carefully estimated based on the entire cell count within the three regions of interest (RI) in every histology slide (cells/mm^2) according to the established standard method (Loqman et al., 2013; Abubakar et al., 2022).

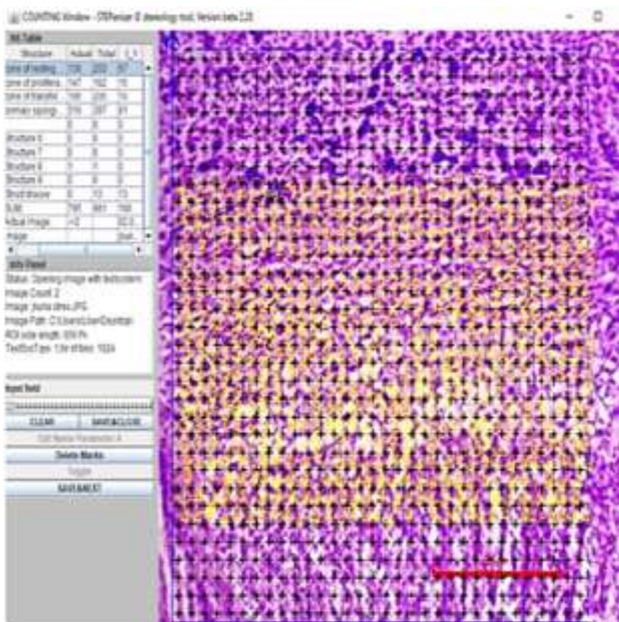

Fig. 2: The analytical computer software used for counting chondrocytes at different zones of growth plates.

Immunohistochemistry

The immunoperoxidase (IP) special staining was performed based on the IHC kit Immuno Cruz™ (Santa Cruz Biotechnology, Inc., USA) according to an existing protocol described by Abubakar et al. (2022). The tissue section on the glass slide was washed with xylene to remove the paraffin wax, rehydrated, and washed with phosphate buffer solution. The tissue section was then demarcated with a liquid blocker pen (Daido Sanyo Co., Ltd. Tokyo, Japan). Sodium citrate buffer (10mM) pH 6.0 at 95℃ was used for antigen retrieval for 20 minutes with a laboratory microwave. The endogenous peroxidase from the tissue was quenched by immersing the slides in a phosphate buffer solution incorporated with 0.3% H_2O_2 for 30 minutes. This was followed by incubation o the slides with the primary antibodies (rabbit polyclonal) NHE1 and AE2 against Na^+/H^+ and HCO_3^- at a dilution factor of 1 to 400 ratio overnight at 25ºC in a moisturized enclosure (Rabbit polyclonal IgG to NHE1 [sc-28758] and AE2 [sc-99048]; Santa Cruz Biotechnology, Inc., Oregon, USA). The slides were washed in phosphate buffer solution, then followed by incubation with a biotinylated goat anti-rabbit secondary antibody. The binding to targeted biomarkers was made visible with 3, 3'-diaminobenzidine (DAB) substrate buffer cultured for 9 minute at 37℃. Finally, the slides were re-stained with hematoxylin for 2 minutes and underwent reversed-hydration in increasing alcohol grading.

In the fluorescence immunohistochemistry (FIHC), the bone tissue sections were bleached via exposure to UV light for 40 minutes to remove the tissue's automatic fluorescence prior to de-waxing of the slides. Retrieval of the antigen and the incubation with the primary antibodies were similar to the IP procedures described above. After incubation with the two primary antibodies, the slides were incubated in the secondary fluorescence antibody; goat anti-rabbit IgG conjugated with a dilution factor of 1 to 200 in a dark, moisturized enclosure for 35 minutes at 37℃. This was followed by washing the slides with PBST in the absence of light. The slide was mounted with Dako anti-fade fluorescent mounting medium, and the slide edges of the slides were sealed with nail polish. The immunohistochemistry staining (IP and FIHC) were graded using 5 points scoring criteria adopted from (Fedchenko and Reifenrath, 2014); No tissue reaction (0); tissue reaction less than 5% (1); 5-50% positive tissue reaction (2); tissue reaction greater than 50% with weak stain (3) and tissue reaction greater than 50% with strong stain (4). The grading was conducted by some independent pathologists who were blinded to the experimental design.

Statistical Analysis

The generated data were subjected to a normality test to determine if the data set was parametric or not. Data related to bone length and chondrocyte density were expressed as mean ± SEM, while data generated from immunohistochemistry scoring were expressed as median. Comparisons of data within the treatment groups were made using one-way ANOVA and Kruskal-Wallis tests where applicable. Statistical tests were performed using SPSS version 22.0. Turkey post hoc was used to determine statistical significance when $P<0.05$.

RESULTS

Membrane Inhibitors' effect on the Bone Length in the Presence of Growth Hormone

In order to investigate the role of the Na^+/H^+ exchanger (NHE1) and HCO_3^- anion exchanger (AE2) membrane transport on bone lengthening in the presence of recombinant growth hormone (rhGH), intact metatarsal and tibial bones were incubated in a standard culture media for 48 hrs ex vivo. The metatarsal and tibial bones were separately treated with either the growth hormone alone (rhGH), a combination of the growth hormone with EIPA (rhGH + EIPA), a combination of growth hormone with DIDS (rhGH + DIDS) or the DMSO vehicle that was used to constitute EIPA and DIDS powder (DMSO). The bone lengths were measured before incubation and after

48 hours of incubation (Table 2). Significant differences in bone lengthening were recorded after 48 hours of incubation among the treatment groups (P<0.05) compared to the baseline values at 0 hours. However, no significant differences (P>0.05) were observed between the EIPA + rhGH and DIDS + rhGH groups in both metatarsals and tibial bones lengthening, suggesting that, in the presence of the growth hormone, the plasma membrane inhibitors under investigation (EIPA and DIDS) can significantly suppress the whole bone growth (Fig. 3).

Table 2: Mean metatarsal and tibial length at baseline and after 48 hours of incubation period

Treatment	Metatarsal		Tibia	
	Baseline length (cm)	Length after 48h (cm)	Baseline length (cm)	Length after 48h (cm)
DMSO vehicle (n=5)	0.84±0.01	1.03±0.02	1.94±0.12	2.32±0.05
rhGH (500ngmL^{-1}; n=5)	0.84±0.01	1.10±0.02	1.98±0.04	2.44±0.05
EIPA + rhGH (444µM+500ngmL^{-1}; n=5)	0.81±0.01	0.96±0.02*	1.98±0.01	2.19±0.04*
DIDS + rhGH (250µM+500ngmL^{-1}; n=5)	0.86±0.01	0.95±0.02*	1.97±0.02	2.23±0.05*

Data were generated from the two tibia and the three middle metatarsal bones, from the 20 rats, with each group containing a minimum of fifteen metatarsals and ten tibial bones. Bone length measurements were taken before and after incubations at 48hr with measuring software as indicated in materials and methods. *Denotes significant difference from the rhGH control (one-way ANOVA

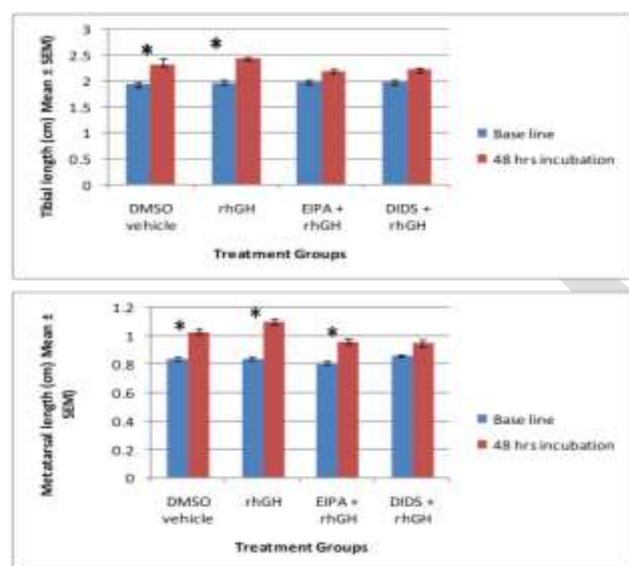

Fig. 3: Mean bone length (cm), variation between bone growth at baseline (0hr) and after ex vivo incubation (48hr) in all treatment groups. The data were generated from the two tibial and three middle metatarsal bones of 20 rat pups, with each group containing a minimum of fifteen metatarsals and 10 tibial bones. *denotes significant differences between baseline data and their corresponding 48 hours incubation periods.

In both metatarsal and tibial bones, treatment with rhGH had the highest growth recorded during the 48hr incubation period, followed by those treated with DMSO vehicle alone, but no significant difference was noticed between the rhGH treated and DMSO treated control. However, there were significantly lower growth differences among the rhGH + EIPA, and rhGH + DIDS groups, with both rhGH + EIPA and rhGH + DIDS having lower growth rates (Tibia; 2.19±0.04cm, Metatarsal; 0.96±0.02cm), (Tibia; 2.23±0.05cm, Metatarsal; 0.95±0.02cm) respectively, when compared with the rhGH treated group alone (Table 2). The lowest bone growth was recorded in rhGH±EIPA (Table 2; Fig. 3), which suggests that EIPA inhibition was more effective than DIDS.

The tibia and metatarsal bone percentage growth rate were expressed as percentage length changes (Table 3). The percentage bone length increase was higher in rhGH treated group, tibia (18.86±2.13); metatarsal (23.30±1.97%). The lowest percentage bone growth increase was recorded in rhGH+DIDS group (11.62±1.70; 9.38±1.65%) tibia and metatarsal respectively (Table 3). There were significant differences in percentage bone growth increase (P<0.05; one way ANONA) among rhGH, rhGH+EIPA and rhGH+DIDS groups, indicating that percentage increase in bone growth was negatively affected in the membrane inhibitor treated groups, even though in combination with growth hormone. There was no significant difference in percentage bone growth increase between the DMSO vehicle and rhGH-treated groups, even though the rhGH percentage bone growth was higher than that of the DMSO vehicle (Table 3).

Table 3: Mean bone percentage growth rate in various experimental treatments 48 hours after incubation

Treatments	Metatarsal % change	Tibial % change
DMSO vehicle	18.12±1.93	15.63±5.54
rhGH (500ngml^{-1})	23.30 ± 1.97	18.86 ±2.13
EIPA + rhGH (444µM+500ngmL^{-1})	14.75±2.11*	9.06±1.92*
DIDS + rhGH (250µM+500ngmL^{-1})	9.38±1.65*	11.62±1.70*

Mean Metatarsal and tibial length percentage change after 48hr ex vivo incubation in all the groups. Data were generated from the tibia and the three middle metatarsal bones from the 20 P10 rat pups. Percentage bone length changes were expressed from bone lengths after 48hr ex vivo incubation. *Significantly different from the corresponding positive control (rhGH) (P<0.05; one-way ANOVA).

Growth Plate (GP) Length Inhibitory Changes

Total growth plate (TGP) length and the hypertrophic zone (HZ) length of proximal tibial bone were determined from the histologic slides (Fig. 4), percentage hypertrophic zone length was calculated from the entire GP length. Hormone treated group had the highest TGP length, followed by the DMSO group (655 ± 43.45; 631.51 ± 32.50 µm), while the lowest TGP length (535.44 ± 50.22 µm) was recorded in the rhGH+DIDS group (Table 4 and Fig. 4). One-way ANOVA showed that there were no significant TGP length differences among the treated groups compared to the rhGH group, suggesting that all the groups maintained relatively constant TGP length growth during 48hr ex vivo incubation. However, the HZ length of the GP showed great growth variation (Fig. 4).

The pattern of HZ growth was also similar to that of TGP, with rhGH having the highest growth length followed by DMSO whiles the rhGH+DIDS has the lowest growth (Table 4). There was significant HZ growth changes (P<0.05, ANOVA) within rhGH and groups with inhibitors EIPA; DIDS in the presence of rhGH, but no significant difference observed between HZ length of rhGH and DMSO groups. This may indicate that the growth in HZ length is the major determinant of the total bone growth in length. The percentage growth rate of the HZ length of the TGP also showed significant variations among the different treatment groups, except between rhGH and DMSO as previously recorded in HZ length (Table 4).

Proofs

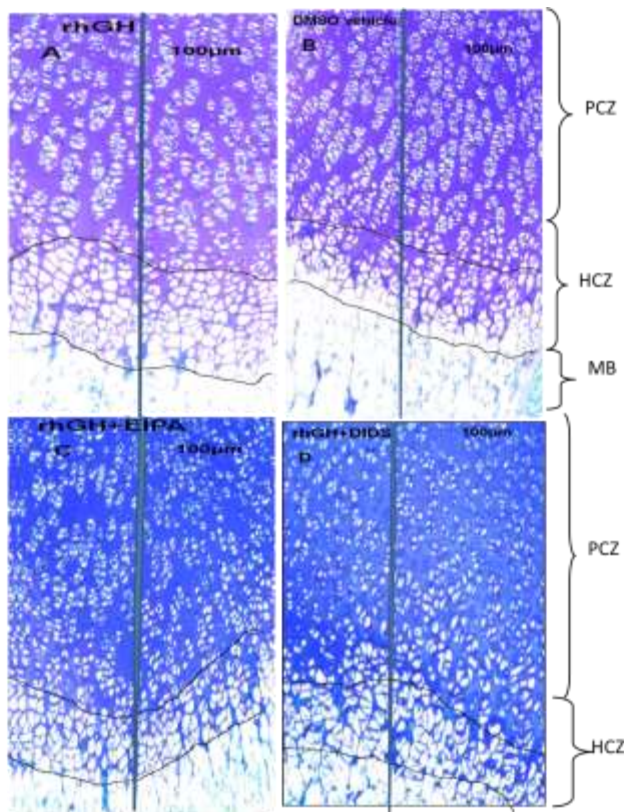

Fig. 4: Total growth plate height and HZ GP length in various treatment groups. Following 48hr exposure with different treatments. The bones were fixed and histologically processed and stained with Toluidine blue O. The proximal growth plate was located and the hypertrophic zone chondrocytes were identified by eye between the proliferating chondrocyte region and the mineralized bone (MB) at the bottom. The straight black vertical lines at the mid GP section were used to determine the total length of the GP. PCZ, proliferating zone; HCZ, hypertrophic zone and MB, mineralized zone. Scale bar =100μm in all panels, x10 objective.

Table 4: The Effect of Different Treatments on Tibial Total GP (TGP) and HZ lengths

Treatments	Total GP Length (μm)	HZ Length (μm)	HZ (% of Total)
DMSO vehicle (n=5)	631.56 ±32.50	143.33±5.47	24.33±1.75
rhGH (500ngmL^{-1}; n=5)	655.00±43.45	153.44±2.87	25.73±1.84
EIPA + rhGH (444μM+500ngmL^{-1}; n=5)	552.78±27.32	130.22±6.02*	23.86±1.35*
DIDS + rhGH (250μM+500ngmL^{-1}; n=5)	535.44±50.22	128.89±4.69*	23.70±1.52*

Proximal tibias from (n=20); P10 rats were cultured for 48hr in various treatments at the concentrations indicated in the table. Bones were fixed, prepared, and visualized. Total GP and HZ lengths were measured after histological sectioning of the bones; HZ length was expressed as percentage length of total GP length. *Denotes significant difference from the corresponding positive control (rhGH) (P<0.05; One-way ANOVA)

Growth Inhibitory Effects on Growth Plate (GP) Chondrocytes Population

Total growth plate (TGP) chondrocytes and hypertrophic zone chondrocytes densities were determined from the histologic sections of proximal tibial bone as described in the materials and methods (Table 5). The rhGH-treated group had the highest chondrocyte population within the entire GP length, followed by the DMSO group (2957.60±64.02; 2408.60±20.50 cells/mm^2), while the lowest chondrocyte population was recorded in rhGH+DIDS. There were also significant differences among the experimental groups in comparison with the rhGH treatment, but no significant difference was observed between the rhGH and DMSO groups (One-way ANOVA). However, the chondrocyte density within the hypertrophic zone seemed to have maintained a relatively constant population, and the results showed that there was no significant chondrocytes density among the treated groups, even though a similar spattern of density was observed as recorded in the entire GP length, with the rhGH and DMSO having the highest chondrocyte population, while the rhGH+EIPA and rhGH+DIDS have lowest density, as shown in Table 5. This finding may also suggest that chondrocyte population within the HZ of the GP may not be a determinant of the zone length.

Table 5: The Effect of Different Treatments on Tibial Total GP and HZ Chondrocytes Densities

Treatments	Total GP Cell Density (Cells/mm^2)	HZ Cell Density (Cells/mm^2)
DMSO vehicle (n=5)	2408.60±20.50	1121.60±15.71
rhGH (500ngmL^{-1}; n=5)	2957.60±64.01	1370.40±19.92
EIPA + rhGH (444μM + 500ngmL^{-1}; n=5)	2179.20±34.99*	1057.60±34.91
DIDS + rhGH (250μM + 500ngmL^{-1}; n=5)	2162.60±21.08*	1062.80±14.08

Proximal tibias from (n=20); P10 rats were cultured for 48 hrs in various treatments at the concentrations indicated above. Bones were fixed, prepared, and visualized. Total growth plate (GP) and hypertrophic zone (HZ) cell densities were quantified using software after histological sectioning of the bone. *Significantly different from the corresponding positive control (rhGH) (P<0.05; One-way ANOVA).

Immunoperoxidase and Immunofluorescence-Associated Expression of NHE1 and AE2 in the Presence of Growth Hormone

The immunoperoxidase and immunofluorescence localization of NHE1 and AE2 membrane transport were graded 48hr after ex vivo incubation of the bones. Proximal growth plate sections were stained with NHE1 and AE2 primary antibodies to determine cell-associated expression of NHE1 and AE2 in the growth plate. The immunoperoxidase and immunofluorescence reactive expression was graded and compared in all the experimental groups (Fig. 5). Hormone-treated group has the highest expression of both AE2 and NHE1 proteins, followed by DMSO (3.75±0.13; 3.5±0.20) out of the maximum score of 4, the least expression score was recorded in rhGH+EIPA group (Fig. 5). There was a significant difference among rhGH, rhGH+EIPA and rhGH+DIDS, but no significant difference exists between the rhGH and DMSO groups, this suggests that plasma membrane inhibitors have a significant inhibitory effect on the expression of both the NHE1 and AE2 proteins, even in the presence of growth hormones. The inhibitory effect of EIPA was higher when compared to that of DIDS (2.58±0.26; 2.92±0.32) due to the low expression of the membrane proteins in rhGH+EIPA group, even though there was no significant difference observed (Fig. 5).

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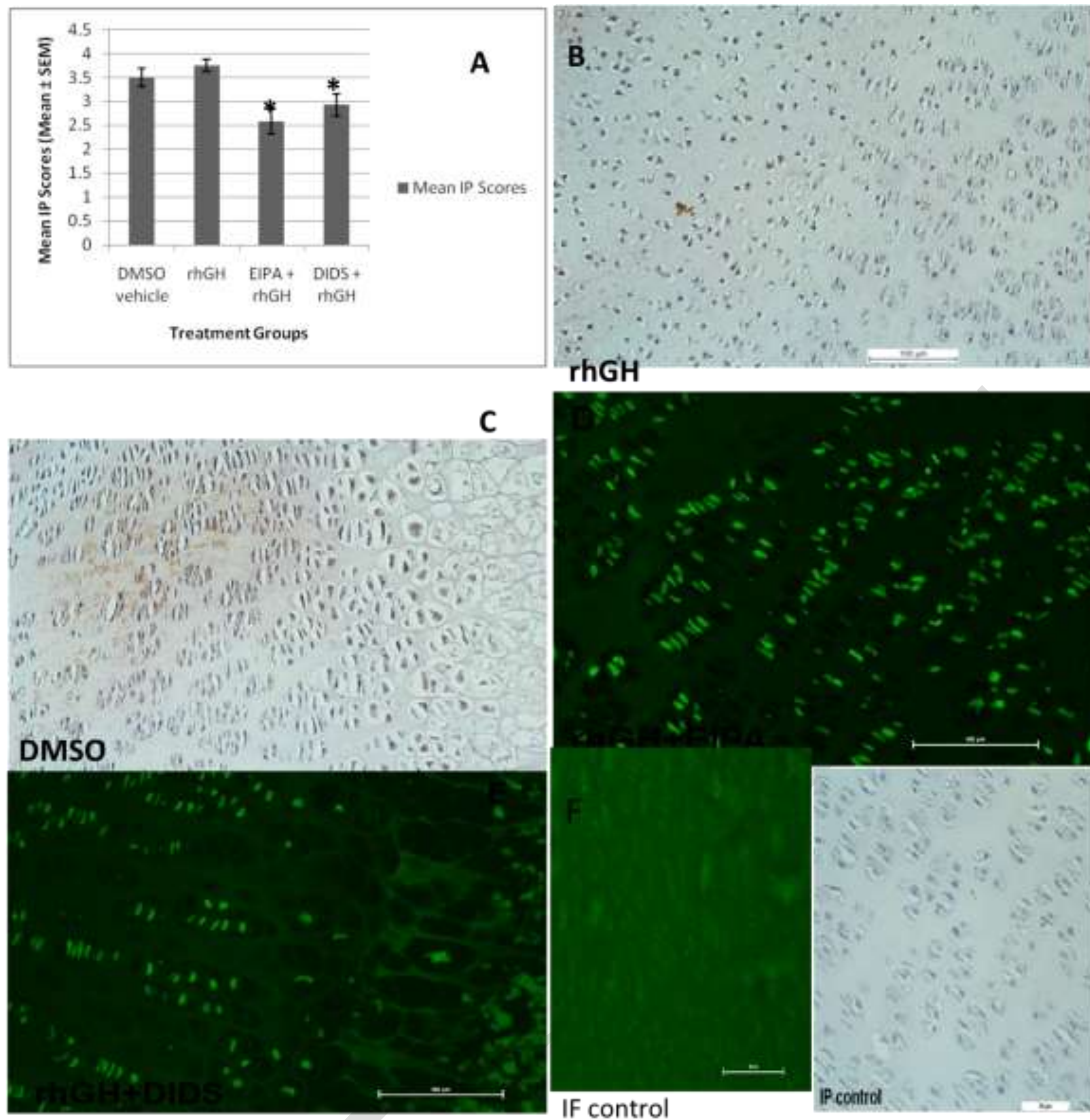

Fig. 5: The immunoperoxidase (IP) scores result (A), appearance of the IP and immune-fluorescence (IF) expression in proximal tibial GP chondrocytes at different zones after 48hr culture with rhGH alone (B), DMSO vehicle alone (C), IF stained rhGH+EIPA (D), IF rhGH+DIDS (E) and primary antibody negative control of IP and IF. Tibial bones were cultured in various treatments as indicated in the materials and methods for 48 hours and labeled with NHE1 and AE2 antibodies. Antibody expression was detected with DAB chromogen stain in slides B, C, and IP control (IP) slides, while conjugated fluorescence secondary antibody was used to detect target antigen expression in IF slides (D, E, and IF control). Chondrocytes were then visualized by fluorescence microscope at regions of interest. *denotes significant mean IP score differences among rhGH, rhGH+EIPA and rhGH+DIDS treatments. Scale bar =100μm in all panels, x10 objective.

DISCUSSION

The mammalian long bone growth process is complex, as it is usually influenced by nutritional, neuronal, and hormonal factors that are necessary for the growth and development of the bone (Menagh et al., 2010; Wit and Camacho-Hübner, 2007; Dua et al., 2021). Several hormonal activities are required for postnatal normal somatic bone growth, but growth hormone (GH) is the only one among others that can trigger bone growth lengthwise in a dose-dependent manner through the regulation of protein, carbohydrate, and lipid metabolism (Tritos and Klibanski, 2016; Zhao et al., 2025). The exact mechanism by which GH exerts its stimulatory effects on linear bone growth is not fully understood, but it was reported by Carter-Su et al. (2015); Luo et al. (2024) that it stimulates the process of chondrogenesis, which subsequently brings about bone elongation indirectly via increasing production of the (IGF-1).

The ex vivo longitudinal growth of metatarsal and tibial bones from P10 rats in the presence of recombinant human growth hormone (rhGH) were significantly retarded following treatment with the investigated plasma membrane inhibitors (EIPA and DIDS). EIPA and DIDS have been reported to be extensively used against the respective membrane transport of NHE1 and AE2 in numerous cell types, including the brain, kidney, liver, heart, exocrine, and many other cell types (Gonzalez-Begne et al., 2007; Menagh et al., 2010; Coppini et al., 2013). The two plasma membrane inhibitors (EIPA and DIDS) were recently reported to suppress mammalian longitudinal bone growth in a dose dependent manner

through a hypertrophic chondrocyte regulatory volume mechanism (Loqman et al., 2013; Abubakar et al., 2022). To date, there is no established data reporting the effects and physiological roles of EIPA and DIDS in the presence of growth hormone during ex vivo long bone growth. We, therefore, make an attempt to inhibit metatarsal and tibial bone growth ex vivo in the presence of recombinant growth hormone (rhGH) at a concentration of 500ngmL^{-1} to establish if hormonal physiological influence may continue to occur in the presence of plasma membrane inhibitors of NHE1 and AE2.

Remarkable increase in whole bones, total GP length, and total GP chondrocytes densities were recorded in the rhGH-treated group in comparison with the control group treated with DMSO vehicle alone, suggesting synergistic effects of the growth hormone in bone growth. Although there were no statistically significant differences between the rhGH and DMSO groups in all the parameters, the results suggested that ex vivo bone growth in the presence of GH does not have a direct stimulating effect on bone growth, but there could be an indirect metabolic effect of the growth hormone on bone lengthening ex vivo. The increase in bone length in the rhGH-treated group could be associated with active increase in chondrocytes proliferation and differentiation along the entire GP length due to indirect metabolic effects of the rhGH, as seen in Table 5, the total GP chondrocytes count was higher in the rhGH groups. Many authors also reported that GH can directly bind to its target receptors to stimulate mitotic cell activities without necessary mediation by IGF-1 (Locatelli and Bianchi, 2014; Lindsey and Mohan, 2016; Tritos and Klibanski, 2016; Yang et al., 2020). This suggests that GH seems to have a direct effect on bone growth by stimulating the differentiation of chondrocytes, a similar finding reported by the researchers cited above.

The two inhibitors (EIPA and DIDS) appear to have similar patterns of bone growth inhibition in the presence of rhGH, as both pharmacological agents significantly suppressed the gross length of the bones in a relatively similar manner. In rhGH+EIPA and rhGH+DIDS treated groups, the growth variation between the bone at baseline before incubation and 48 hours after incubation was not significant, suggesting that whole bone growth was significantly suppressed by the pharmacological inhibitors of the NHE1 and AE2 in the presence of the rhGH. EIPA appeared to have a significant inhibitory effect on the growth of both metatarsal and tibial bone compared to DIDS. This finding strongly indicates that NHE1 membrane protein does play a crucial role during endochondral bone formation as reported by Abubakar et al. (2022).

Considerable inhibition of GP length was recorded in the proximal tibial bone when exposed to EIPA and DIDS in the presence of rhGH, but no statistically significant differences among the treatment groups. This finding suggests that the ex vivo linear growth of bone enhanced by rhGH is not determined by the total GP length growth. The finding may also indicate that the major contribution to long bone growth is probably restricted to the physiological roles played in certain regions of the GP, but not in the entire growth plate length. It has been reported that the hypertrophic chondrocyte region of the growth plate is the most important determinant of long bones (Loqman et al., 2013; Sun and Beier, 2014; Abubakar et al., 2022; Zhao et al., 2025). Remarkable changes in GP HZ length were observed within the various treatment groups, which may suggest that the hypertrophic chondrocytes area is the most important contributor to linear bone growth. This is consistent with the established reported data (Studer et al., 2012; Loqman et al., 2013; Abubakar et al., 2019; Yao et al., 2021). This study also demonstrated that there was no considerable difference in the hypertrophic chondrocyte population within the various experimental groups when compared to the DMSO control group. This strongly indicates that even though there was a significant length variation in the hypertrophic chondrocyte zone, the chondrocyte population within the zone remained relatively constant. This finding suggests that chondrocyte volume increase could be responsible for bone lengthening, as the chondrocyte densities within the hypertrophic zone were relatively constant in all the treatment groups. Our previous study (Abubakar et al., 2022) has implicated hypertrophic chondrocyte volume increase as the major determinant of bone lengthening. The proposed increase in volume of hypertrophic zone chondrocytes in the both rhGH and DMSO groups that were not treated with either of the inhibitors might have occurred through regulatory volume increase (RVI) due to physiological exchange of the two plasma membrane proteins investigated (NHE1 and EA2) across the plasma membrane of the chondrocytes, which subsequently bring about changes in intracellular pH that initiate chondrocyte volume increase via extracellular fluid absorption, hence the increase in hypertrophic zone length observed in this study.

The significant inhibitory variation of chondrocyte densities recorded in the HZ among the rhGH+EIPA and rhGH+DIDS groups could also suggest that NHE1 and AE2 protein movement across chondrocyte membrane has taken place along the entire growth plate length. The NHE1 and AE2 proteins were highly expressed within the entire growth plate length in the control groups (rhGH and DMSO). Significantly decreased cellular expression and fluorescence signaling in the rhGH+EIPA and rhGH+DIDS treated groups was also recorded. However, NHE1 protein expression and associated fluorescence associated signaling was observed to be higher than AE2 membrane proteins in the control groups. This also signified a greater role of NHE1 in bone lengthening when compared to AE2, which supports our previous assertion that implicates NHE1 is the major player in overall bone lengthening. The remarkable suppression of NHE1 and AE2 expression was more pronounced in the hypertrophic zone of chondrocytes, in comparison to the chondrocyte proliferation region. This strongly indicates that the expression of NHE1 and AE2 is more crucial in the hypertrophic chondrocytes zone to facilitate bone growth than in other zones of the growth plate.

This study utilizes the conventional ex vivo bone culture for a period of 48 hrs to monitor changes in the growth plates and overall bone growth. The ex vivo

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incubation period should have been extended beyond 48 hours for a better understanding of the effects of the growth hormone concurrent with the membrane proteins during bone growth. Using a bioreactor for the ex vivo culture technique, if available, will help address this limitation.

Conclusion

The recombinant growth hormone appeared to have no direct effect on bone growth, but may have an indirect metabolic stimulating effect via differentiation of the chondrocytes along the growth plate. EIPA and DIDS, plasma membrane inhibitors, can still suppress longitudinal bone growth in the presence of growth hormone. In the presence of growth hormone, the expression of NHE1 and AE2 membrane proteins was enhanced, which facilitate longitudinal bone growth. NHE1 and AE2 plasma membrane proteins play a crucial role in bone lengthening, independent of growth hormone. Further studies need to be conducted to explore the expression of insulin-like growth factors and other proteins that regulate the physiological functions of the growth hormone in the growth plate. Further studies should also look into the potential downstream signaling pathways and cellular interactions that are regulated by NHE1 and AE2 in the presence of growth hormone.

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Conflict of Interest: To the best of our knowledge, there was no conflict of interest whatsoever.

Data Availability: The data presented in this research are available from the corresponding author upon reasonable request, provided it will be appropriately utilized.

Ethics Statement: The approval for the experiment was granted by the Universiti Putra Malaysia committee for experimental animal use (ICUC) ref. no: R028/2015. The animal experimentation was conducted in compliance with the Malaysian Code of Practice for the Care and Use of Animals for Scientific Purposes as recommended by ARRIVE guidelines.

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