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Original Article

Hylonome

Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve pre-cavitated joints

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Abstract

The influence of movement on joint space formation during limb development has been the subject of much interest. Our aim was to investigate the short-term influence of movement upon cavitation by immobilizing chick embryos *in ovo*, both in a rigid manner where dynamic stimulation is removed, and a flaccid manner where both dynamic and static stimulation are absent. Induction of rigid immobilization with decamethonium bromide (DMB) or the novel induction of flaccid immobilization with pancuronium bromide (PB) for 3 days, during the normal cavitation of joints resulted in the loss of cavity formation. Immobilization after the formation of an overt cavity demonstrated that static stimulation (during rigid paralysis) was able to maintain joint cavities and preserve some of the hyaluronan (HA) content of articular surfaces, whereas flaccid paralysis resulted in the loss of cavities and a marked depletion of HA content. Assessments of the growth and deposition of cartilage and bone in the limbs of embryos immobilised during cavitation showed that the length of limb elements was greatly reduced and that decreases in epiphyseal widths were most marked and more pronounced distally. The volume of bone in these elements remained unchanged whereas the cartilage volume decreased significantly, suggesting that chondrogenic but not osteogenic events in the embryo are particularly sensitive to mechanical stimulation. In addition to describing a novel method of inducing flaccid immobility *in ovo*, these data point towards the important role of both static and dynamic stimuli in the growth of embryonic limbs and the development of a functional joint space.

Keywords: Immobilization, Hyaluronan, Chick, Joint, Cavity

Introduction

Establishing separation between developing articular ends of discrete elements in the embryonic limb is a vital event during joint formation and skeletogenesis. The earliest commitment of cells to such a fate is evident in areas of blastemal mesenchyme found interposing each pair of developing cartilaginous condensations[1-3]. As limbs develop, these cartilaginous regions (anlagen) grow and the intervening presumptive joints persist as densely packed regions of flattened cells (interzone) that become attenuated by virtue of their limited expansion. Despite recent identification of factors that regulate joint formation and exhibit selective expression in presumptive joint regions[4,5], the mechanisms responsible for facilitating the development of synovial articular joint cavities (cavitation) remain to be fully determined.

The cavitation process does not appear as earlier consensus, or as recent reports suggest, to be merely a liquefaction of ground substance or cells, nor cellular retraction or death that allows adjacent spaces to form a composite large acellular space. Rather, it involves a series of supplementary and continuing changes in local differentiation that lead to the joints' distinct range of connective tissues[4,6,7]. We and others have found that during cavitation, which is a relatively late event in limb development, there is localised enrichment in hyaluronan (HA) in the extracellular matrix (ECM) at sites of the presumptive separation, whilst the closely opposed cartilaginous elements retain high levels of sulphated glycosaminoglycans[8-11].

The initial patterning, during which future joints are spec-

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ified, appears to be intrinsically regulated and independent of growth in the adjacent elements. However, it has been proposed that differential rates of tissue expansion may engender local mechanical forces that act to establish lines of separation during the later stage of joint development[12]. Consistent with this proposal it has also been concluded that cavitation, but not initial specification of joints is dependent upon muscular activity[13-19].

Indeed, many studies into embryonic diarthrodial joint development have shown that prolonged lack of muscular movement, results in the fusion of the opposed joint elements and the absence of cavities[14-20]. Cultured embryonic limbs also fuse, suggesting that movement induces formation of the joint space and also facilitates development of shape and structure of the articular surface[16]. Thus, *in ovo* administration of decamethonium bromide (DMB), a depolarising neuromuscular blocker that induces rigid limb paralysis results in the fusion of joint elements[15,19]. Interestingly, administration of the non-depolarising drug, pancuronium bromide, to premature human infants in intensive care in order to produce flaccid paralysis has been shown to result in reduced manually applied hip and knee joint flexion and ankle joint dorsal extension. Thus, even in neonates, paralysis reduces joint mobility and spontaneous muscular activity is necessary to prevent effects of long-term contracture[21]. However, the effects of pancuronium bromide on embryonic chick limb development have not previously been examined.

The embryonic chick, Gallus gallus is an established model for developmental studies, in which joint morphogenesis closely resembles that of humans and as birds are oviparous, embryonic manipulation is relatively easy and non-invasive. Further, the temporal and spatial sequence of events involved is precisely described and documented[15,22-27]. Therefore it is possible to examine distinct phases of cavitation in selected joints and to evaluate the effects of defined periods of paralysis during this process (Figure 1).

Nonetheless, the precise nature of the mechanical stimuli responsible for either initiating joint cavity formation, or retaining functionally appropriate joint surface integrity in developing limbs, remains to be defined. Moreover, the relationship between such events and any immobilization-induced changes in radial or longitudinal growth of the skeletal elements has yet to be determined. In this study, we examine the effects of pharmacological manipulation of embryonic chick skeletal movement with drugs that induce rigid paralysis (DMB) and describe the novel use of pancuronium bromide (PB) *in ovo* to induce flaccid paralysis. We report that short-term rigid or flaccid paralysis results in a loss of joint cavity formation. Although rigid immobilization after overt cavity formation is able to maintain the joint cavity, flaccid immobility is not. Both types of immobilization cause a shortening and narrowing of limb elements with the greatest reductions seen in distal element epiphyseal widths. Cartilage volume of immobilized elements are greatly reduced while bone volume remained unchanged suggesting that cartilage development is more dependent on mechanical stimulation than the development of bone. Therefore this report for the first time compares the short-term effects of rigid and flaccid immobility on the developing limb.

Day	8	9	10	11	12	13	14	15
Cavitation		Stifle		TT	MTP			
Stage	34	35	36	37	38	39	40	41

Figure 1: Diagram showing days of incubation alongside developmental stage of the embryonic chick. The stages at which cavitation begins in the hind limb stifle, tibiotarsal (TT) and metatarsolphalangeal (MTP) joints are indicated.

Materials and methods

All reagents were from Sigma (Poole, England) unless otherwise stated.

Fertilised white leghorn eggs were 'windowed' according to standard protocols[28]. In normal chicks, cavitation of joints proceeds in a proximal to distal manner with cavitation of the stifle (S), tibiotarsal (TT) and metatarsophalangeal (MTP) joints beginning at stage 35 (day 9), stage 37 (day 11), and at stage 38 (day 12) respectively (Figure 1)[29].

Induction of rigid paralysis

Stage 36 or 39 (a stage before overt cavitation of S, TT, and MTP and a stage after cavitation of K and TT joints, respectively) embryonic chicks were treated with sterile-filtered decamethonium bromide (DMB Sigma D1260) at 5mg/ml in Tyrode's solution (TS: 0.8% NaCl, 0.02% KCl, 0.005% $NaH_2PO_4.H_2O$, 0.1% glucose, 0.1% $NaHCO_3$ at pH 7.4) on the first day of treatment (day one), and at 1mg/ml daily thereafter until stage 39 or 41, respectively. Each 100µl dose was aseptically injected through the window of the egg directly onto the chorioallantoic membrane, and melted wax was used to seal the window. On the final day of treatment, chicks were killed by decapitation (Schedule 1, Animal Scientific Procedures Act 1986). A control group of chicks received equivalent volumes of Tyrode's solution alone for the duration of the experiment.

Induction of flaccid paralysis

Stage 36 or 39 chick embryos were treated with 100µl doses of 8mg/ml filter-sterilised pancuronium bromide (PB Sigma P1918) in Tyrode's solution, on day 1 with subsequent daily doses of 5mg/ml until stage 39 or 41 (as above).

Assessment of skeletal movement

Eggs were arranged under a dissection microscope (Leica

MZ8) to allow hind limb movements to be viewed. The activity of each embryo was recorded on videotape for 5 minutes, both before and after the administration of the first 100μl dose of each neuromuscular drug. The duration of any hind limb movement (seconds per minute) was determined from replayed analysis of the video recordings.

Skeletal analysis

After killing by decapitation, the left hind limbs from each group were immediately dissected at the hip joint and immersed in 95% ethanol for 3 days. The cartilage was stained with a solution of 0.15 % Alcian blue 8GX dye (AB; Sigma A3157) in 75% ethanol/20% glacial acetic acid/5% H_2O for 2 days, and then washed in 95% ethanol for 7 days. Tissue was 'cleared' by the addition of 0.5% potassium hydroxide (KOH) for 24 hours and subsequently the bone was stained with fresh 0.5% KOH and 2 drops of saturated filtered Alizarin red S dye (AR; BDH) for 24 hours. Limbs were then processed through fresh solutions of 0.5 % KOH with increasing proportions of glycerol at 50, 70, 90 and 100 %.

The following skeletal dimensions were determined for each element in stained limbs: i) length (femur; F, tibiotarsus; T, tarsometatarsus; TM), distance between proximal and distal articular surfaces; ii) diaphyseal breadth (F, T, TM) at the mid-diaphysis; iii) maximum breadth of proximal (P) and distal (D) epiphyses of stifle (S), tibiotarsal (TT), and metatarsalphalangeal (MTP) joints.

To determine the volume of cartilage (AB) in proximal (P) and distal (D) articular joint regions of S, TT and MTP joints and volume of bone (AR) in each femoral (F), tibiotarsal (T) and metatarsal (MT) element, the area of tissue stained with either Alcian blue or Alizarin red was quantified in images of these stained limbs using the Ofoto programme package, (SmartDraw.com). Limbs from at least three chicks in each treatment group were assessed.

Histological assessment

Embryonic chick hind limbs (at least 3 chicks at each stage) were mounted onto cork, covered with 10% PVA (grade GO4/140, Waker Chemicals, UK) and immersed in n-hexane (grade low in aromatic hydrocarbons, BDH, Poole, Dorset) cooled to -70˚C and then stored at -70˚C[30]. Serial sagittal longitudinal cryostat sections were cut at 10μm using a cabinet temperature of -30˚C, and stored at -70˚C.

To determine whether cavitation was modified by DMB and PB treatment, cryostat sections of the S, TT, and MTP joints from at least three chicks from each treatment group were stained with 0.1% (w/v) Toluidine blue in 0.1 M acetate buffer at pH 6.1. Qualitative assessment of joint formation was determined by comparing the histological appearance of experimental joints to that of the joints in control (TS-treated) chicks at identical developmental stages. Assessment was on a 0-10 scale; 0 denotes non-cavitated or fused joints with the complete loss of articular surface definition and presence of cells with a 'rounded' phenotype in the interzone, 5 denotes the presence of a poorly defined joint space containing 'rounded' cells, and 10 denotes fully cavitated joints with smooth articular surfaces and clear intervening acellular spaces.

Alcian blue staining at critical electrolyte concentrations

The glycosaminoglycan (GAG), hyaluronan (HA), chondroitin and dermatan sulfate (CS and DS) and keratan sulfate (KS), can be distinguished using the cationic copper phthalocyanin dye, Alcian blue (AB). AB binding is electrostatic and dependent on substrate charge density, and on pH and concentration of other cations in the dye bath (Scott and Dorling, 1965). Thus, in the presence of less than 0.2 M $MgCl_2$, HA and sulphated GAGs (sGAGs) are stained. At 0.2 M $MgCl_2$ and above, staining for HA is lost whilst staining for CS, DS and KS is retained.

Accordingly, serial longitudinal cryostat sections were air-dried and fixed in 4% formaldehyde in phosphate buffer, pH 7.4, for 30 minutes and then immersed in 0.025 M acetate buffer containing 0.05% Alcian blue (AB 8GX) and either 0.025 M or 0.5 M $MgCl_2$ at a final pH of 5.8 for 18 hours. Sections were washed three times in corresponding buffer containing $MgCl_2$, dehydrated rapidly and mounted in DPX.

GAG Digestion

Specificity of staining for HA was confirmed by enzymatic pre-treatment of sections prior to AB staining, with 20 U/ml leech hyaluronidase type X (AMS Biotechnology) or 0.5 U/ml Streptococcus dysgalactiae hyaluronidase (AMS Biotechnology) in 50 mM Tris buffer, pH 6.0, for 30 minutes at 37˚C.

Quantification of Alcian blue staining in the developing chick joint

AB staining intensity was measured in interzone (IZ), articular surface layer (AS, fibrocartilage) and epiphyseal cartilage (EC) of both proximal and distal elements in triplicate longitudinal sections (approximately 1 mm apart lateromedially). Staining intensity of at least 3 embryonic chick joints were measured using a Vickers M85A scanning and integrating microdensitometer[31]. Briefly, at least 12 measurements were made in each histologically-defined zone in each section at a wavelength of 550nm using a x40 objective, mask size of A2, scan spot size 1 (0.5 mm) and a 4 second scan time. Measurements of AB staining intensity were made at different $MgCl_2$ concentrations in each zone. These values were used to calculate the relative content attributable to HA, sGAGs and total GAG. Results are expressed as mean integrated extinction (MIE x100 ± standard error of mean, SEM). HA content per unit area was calculated in each of the defined zones as the measured intensity of AB staining at

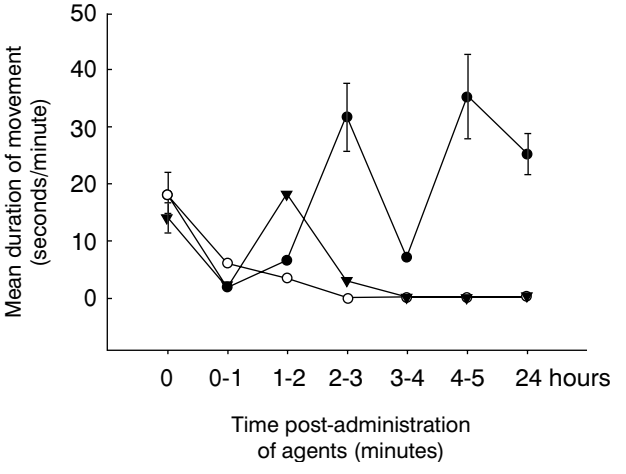

Figure 2: Changes in the duration of limb movement (seconds/minute) at various times after treatment of stage 36 chick embryos with a single *in ovo* dose of neuromuscular drug. Control (Tyrode's solution, filled circles), rigid paralysis induced by DMB (open circles) and flaccid paralysis induced by PB (filled triangles). Data are expressed as mean ± standard error of mean (n=3 for each treatment).

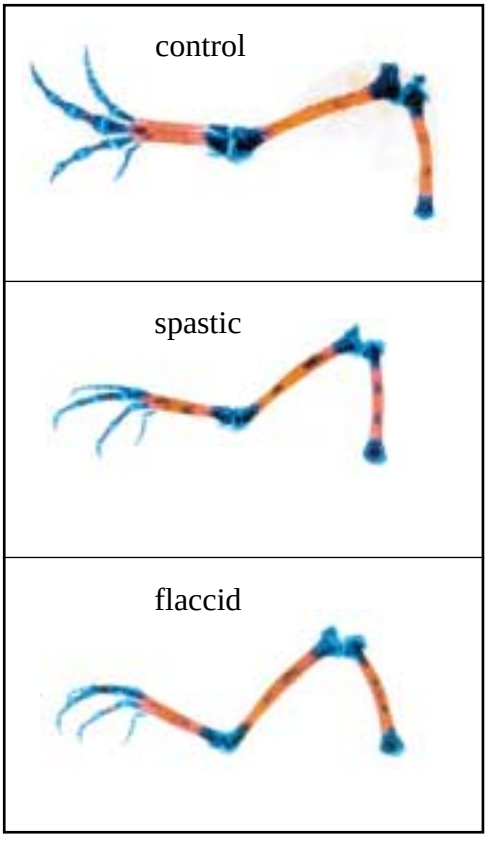

Figure 3: Macroscopic appearance of stage 39 chicks and Alcian blue/Alizarin red-stained hind limbs (stage 41) treated with Tyrode's solution as control, decamethonium bromide to induce rigid paralysis or pancuronium bromide to induce flaccid paralysis.

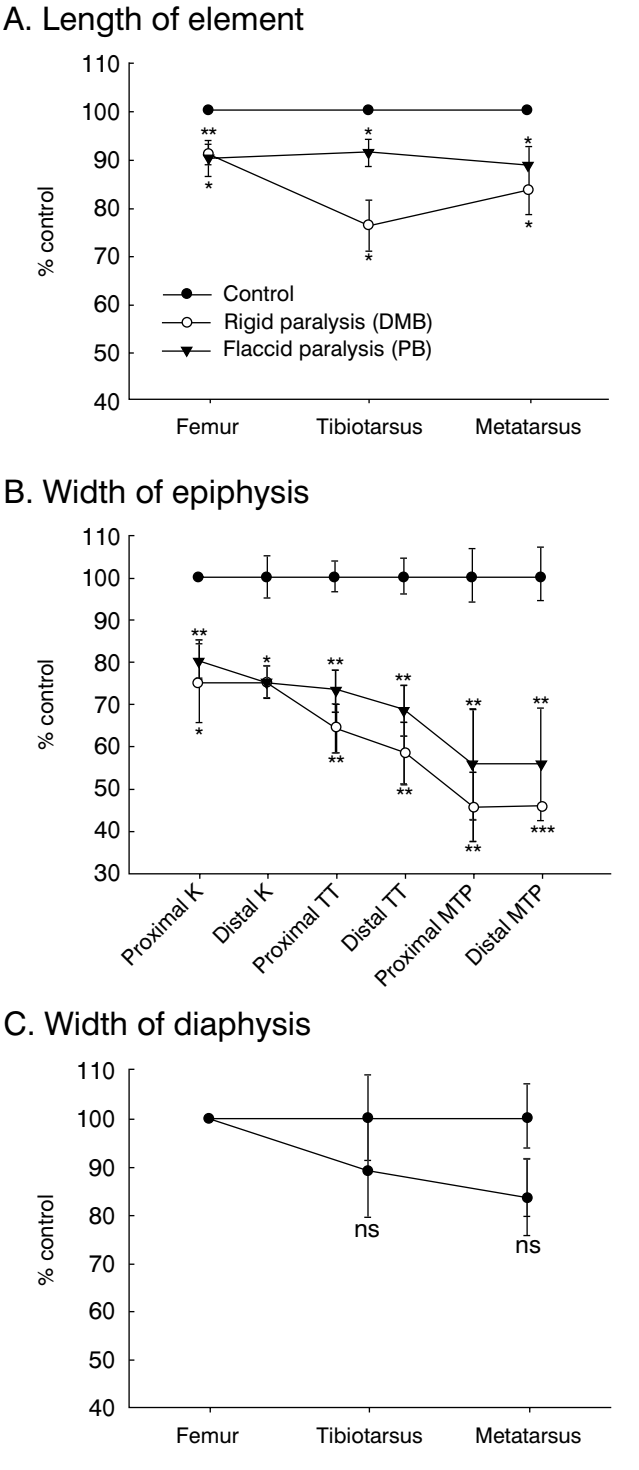

Figure 4: Effect of immobilization at stage 36-39: (A) length of femur, tibiotarsus and metatarsus, (B) epiphyseal breadth in proximal and distal regions of the knee (K), tibiotarsus (TT), and metatarsus (MTP) and, (C) diaphyseal breadth of femur, tibiotarsus and metatarsus. Control embryonic chicks (filled circles), rigidly immobilized chicks (DMB, open circles) and flaccidly immobilized chicks (PB, filled triangles). Results are expressed as percentage of control chick dimensions and each point represents the mean of at least three chicks. Statistical analysis was performed on measurements using the paired Student's t-test where * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

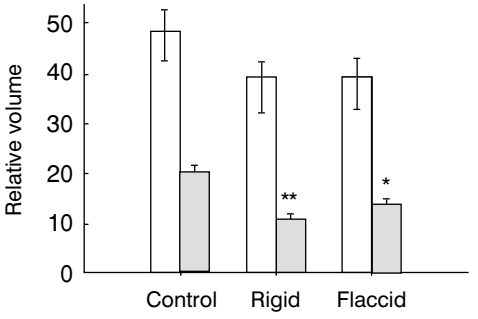

Figure 5: Effect of rigid (DMB) and flaccid (PB) paralysis at stages 36-39 on the relative volume of Alcian blue-stained cartilage (hatched bars) and Alizarin red-stained bone (open bars) in the tibiotarsus. Data are expressed as mean ± standard error of mean for at least three chicks. Statistical analysis was performed using the paired Student's t-test where * denotes $p<0.05$ and ** $p<0.01$.

0.025M $MgCl_2$ (total GAGs), less the intensity measured in the same zone at 0.5M $MgCl_2$. Staining intensity measured at 0.5 M $MgCl_2$ was attributable to sGAG. In some cases, in order to provide a relative HA or sGAG content, these calculated values were expressed as a percentage of total GAG staining (i.e. that measured at 0.025M $MgCl_2$).

Results

In ovo treatment with decamethonium bromide (rigid) or pancuronium bromide (flaccid) induces rapid and sustained immobilization.

Initially, the efficiency of immobilization (stages 34, 36, 39, 41 and 43) that was induced by DMB (rigid) and PB (flaccid) treatment was examined at a range of concentrations (0.1-1.0 mg/day). DMB treatment at stage 43 required 0.5 mg/day, while in less mature stage 34 embryos 0.1 mg/day was sufficient to induce paralysis (not shown). Survival rate was low when DMB treatment commenced at stage 39 (30%), whilst PB treatment produced less morbidity (80% survival). In all subsequent studies, the effect of DMB and PB treatment was examined at stages 36-39 and 39-41, at which times DMB was required at 0.5mg/day, on day one and at 0.1mg/day thereafter. PB was required at 0.8mg/day on day one with subsequent doses of 0.5mg/day, to sustain paralysis.

Skeletal movement in control embryos consisted of variable periods of spontaneous muscular activity (approximately 20 sec/min, range: 10-40 sec/min). At stage 36, treatment with DMB or PB rapidly and completely abrogated skeletal muscular activity and this was sustained for at least 24 hours after this initial dose (Figure 2). We also observed that both DMB and PB decreased embryo heart beat rate; an unexpected finding in PB-treated chicks, as in other species PB increases heart rate by impairing cardiac response to stimulation[32].

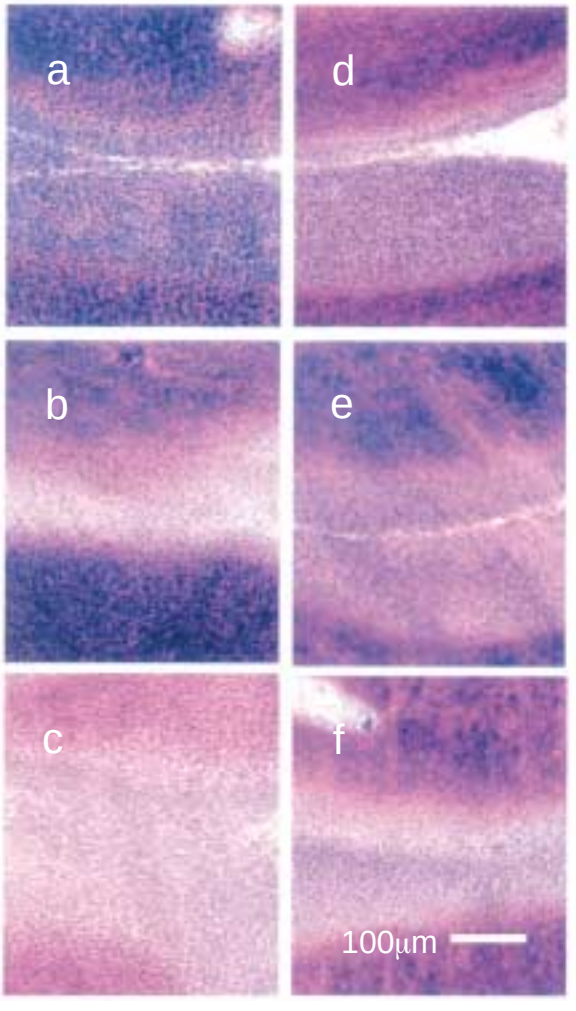

Figure 6: Cryostat sections of TT joints from embryonic chicks treated between stage 36-39 (a-c) and stage 39-41 (d-f) stained with Toluidine blue. Control (a and d); rigidly immobilised (b and e); and flaccidly immobilised chicks (c and f).

DMB and PB have differential effects on embryonic chick posture.

Body postures were significantly modified by DMB and PB treatment. At all stages, 'rigid' DMB-induced paralysis resulted in a contracted-flexed phenotype (Figure 3). In contrast, PB treatment produced relaxed 'flaccid' body postures that lacked muscular tone and had out-stretched limbs. DMB, but not PB, produced severe oedema in the body trunk, and this was more severe at stages 36-39.

Immobilization diminishes radial and longitudinal growth

Whole mount staining of limbs (stage 39) clearly differentiated regions of cartilage and developing bone (Figure 3). Direct measurements showed that both DMB and PB treatment significantly reduced the length of developing femoral, tibiotarsal and metatarsal elements (Figure 4A and Figure 10). This immobilization-induced diminution of growth was

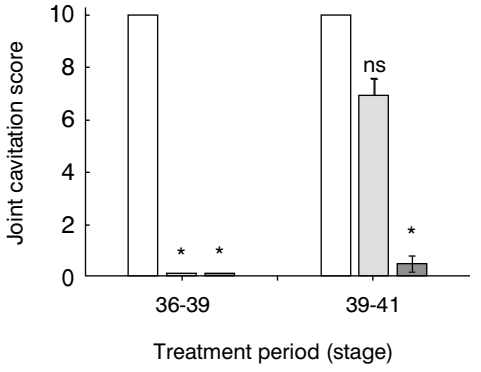

Figure 7: Qualitative assessment of knee joint cavitation in control (open bars); rigidly immobilized (hatched bars) and flaccidly immobilized (cross-hatched bars) embryonic chicks treated between stages 36-39 or 39-41. Assessments were made using the following scale: 0 = joints non-cavitated or fused with complete loss of articular surface definition and presence of 'rounded' cell in the interzone; 5 = Poor joint cavity space with presence of 'rounded' cells; 10 = Joint cavitated with smooth articular cartilage surface and clear joint space. Data are mean ± standard error of mean for 8 chicks per treatment group.

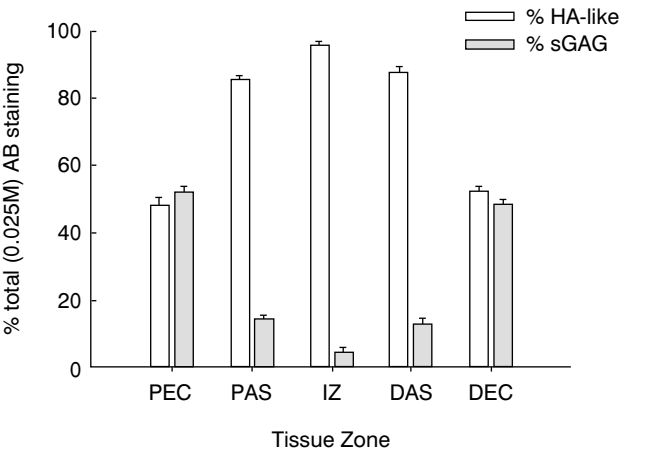

Figure 8: Differential Alcian blue staining for HA-like components in various regions of the tibiotarsal joint. A: Alcian blue staining for HA (0.025-0.5M $MgCl_2$) and sGAGs (0.5M $MgCl_2$) expressed as a percentage of total staining (0.025M $MgCl_2$). (PEC, proximal epiphyseal cartilage; PAS, proximal articular surface; IZ, interzone; DAS, distal articular surface; DEC, distal epiphyseal cartilage).

most apparent at the epiphyses, where there was a greater than 50% reduction in the width of some elements (Figure 4B and Figure 10). This DMB- and PB-induced epiphyseal narrowing was consistently greater in 'less mature' distal elements than in 'older' proximal regions (see Figure 4B and Figure 10), and the effects of DMB were generally more dramatic than those of PB. In contrast, the width of mineralised bone in femoral, tibiotarsal or metatarsal mid-diaphyses showed no significant change in response to DMB or PB treatment (Figure 4C and Figure 10).

Immobilization reduces cartilage, but not bone, volume in the limb.

Rigid and flaccid paralysis at stage 36-39 produced statistically significant decreases ($p<0.01$ and $p<0.05$ respectively) in tibiotarsal cartilage volume compared to control chicks (Figure 5). In contrast, the volume of mineralised bone was unaffected by either form of paralysis (Figure 5).

Rigid and flaccid paralyses inhibit joint cavity formation but exhibit differential effects on maintaining cavity integrity.

As shown histologically, short-term rigid and flaccid paralysis between stages 36-39, which coincides with tibiotarsal-metatarsal (TT) joint cavitation, both resulted in the loss of overt joint spaces (Figure 6 a-c). Similar findings were evident in the metatarsal-phalangeal (MTP) joint (not shown). In these joints, retarded cavity development resulted in cartilaginous 'fusion' without overt menisci or cruciate ligaments (Figure 6 a-c, Figure 7), and a reduction in the size of the patellae (Figure 3). These immobilized TT joints also contained numerous 'rounded' cells at the site of the presumptive joint cavity (see Figure 6).

In flaccidly paralysed chicks at stage 39-41, all the tibiotarsal joint articular surfaces showed evidence of cartilaginous fusion (Figure 6f and Figure 7). At this later stage, after TT joint cavitation is normally complete, rigid paralysis resulted in at least partial retention of all TT joint cavities (Figure 6e and Figure 7), in which the articular surfaces exhibited a 'ragged' appearance compared to control stage 41 joints (Figure 6e and d).

Rigid and flaccid paralyses modify glycosaminoglycan content in a manner related to their cavitation-inhibitory influence.

Consistent with our previous findings[7], relative HA content was greatest in regions bordering the presumptive cavity, comprising up to 95% of total GAGs in the interzone and as little as 50% in the epiphyseal cartilage of the TT joint during cavitation (stage 39, Figure 8). In limbs immobilized before cavitation (stage 36-39), rigid and flaccid paralysis induced identical patterns of decreased HA content in the distinct regions of developing TT joints. DMB and PB both produced statistically significant decreases in HA-like content in regions close to (PAS and DAS, Figure 9A) and within (IZ) the presumptive joint region, without significantly reducing HA content in the epiphyseal cartilage (Figure 9A).

In contrast, limbs that were immobilized after cavity formation (stages 39-41) showed marked differences in the influence of these two forms of paralysis. Rigid paralysis produced small but significant reductions in HA content in both articular surface regions without modifying HA content in the epiphyses. Flaccid paralysis consistently produced more marked diminution in HA content in all regions (Figure 9B).

Enzymatic pre-digestion produced significant loss of HA-like GAG staining intensity in all regions (not shown).

Discussion

This study describes a novel method for pharmacological induction of flaccid paralysis in developing embryos. By comparing the short-term effects of such pancuronium bromide-induced flaccidity to those of rigid paralysis induced by DMB we have found that they exert similar cavitation-inhibitory effects on the process of joint formation, but that they have divergent effects on pre-formed cavity maintenance. We also show that both paralyses modify radial and longitudinal growth in a manner that is most prominent as a diminished epiphyseal width, and reduction in the volume of cartilage, but not bone, and also that these changes are more marked in the distal than proximal skeletal limb elements.

Muscular activity associated with articulation clearly engenders many mechanical stimuli. It produces physiological static loading of joints, by virtue of the sustained contraction of particular muscles, but it also creates dynamic stimuli as a result of their intermittent and discontinuous nature. Defining these model systems for inducing rapid, sustained short-term immobilization will allow the effect of 'dynamic' and 'static' components of this mechanical milieu, on factors that are thought to play roles during joint specification, formation and maintenance to be determined. Their complete removal via use of PB-induced flaccid immobility, or isolation from their dynamic quality via DMB-induced rigid paralysis, will allow the contribution of such parameters to be evaluated. Using such studies we show that 3 days of flaccid or rigid paralysis are sufficient to halt the histological appearance of joint cavities. Both also reduce local HA content, supporting a role for local HA accumulation in the cavitation process.

Movement has been described as essential for functional joint cavity formation. Thus, secondary structures including the cavity and the articular cartilage are dependent on skeletal movement (unlike the fundamental regulation of joint shape and organisation)[18,23,33]. Consistent with this we find that short-term flaccid and rigid immobilisation of chick limbs, before or during cavitation (stage 36-39) results in failed joint cavitation; with articular surfaces, menisci and ligaments also failing to develop. The similarity in the pre-cavitational influence of these two distinct forms of paralysis is contrasted with their effect upon cavity maintenance which appears to differ; with more pronounced histological regression and diminished HA content in pre-cavitated joints subjected to flaccid paralysis. This suggests that the static component of loading, which is sustained by rigid paralysis, makes a unique contribution to maintaining cavity integrity. Further, the association between such influences and local HA content also endorses the likely role for HA in these events. Previous studies using myogenin deficient mice have shown normal cavitation in circumstances where contracting skeletal muscle has failed to form, and it is therefore possible that maternal movements are sufficient for embryonic joint development in mammals[34].

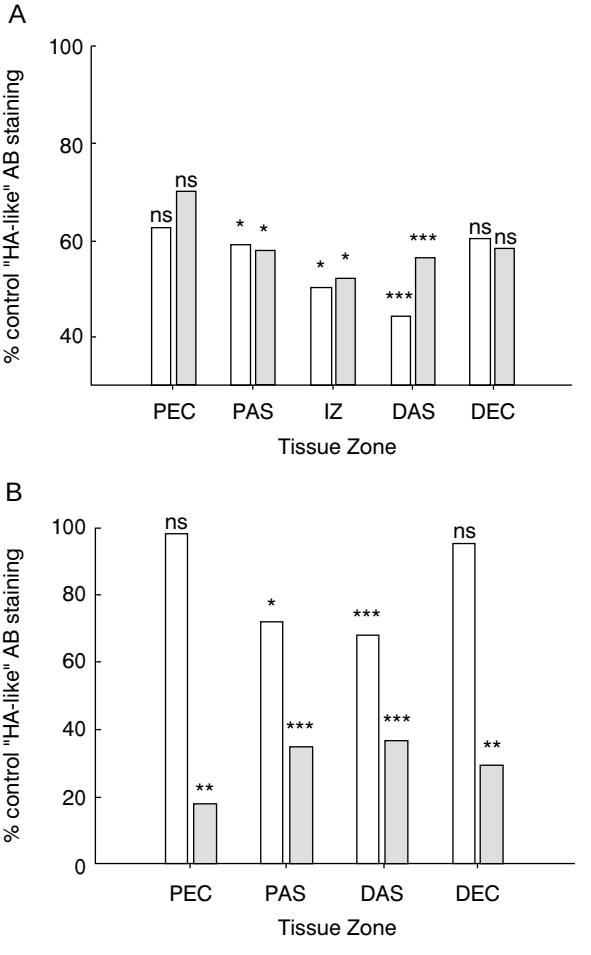

Figure 9: A and B: Effect of rigid (DMB, open bars) and flaccid (PB, hatched bars) paralysis on HA content (percentage of control) in various regions of the developing tibiotarsal joint during (stages 36-39, B) and after (stages 39-41, C) cavity formation. (PEC, proximal epiphyseal cartilage; PAS, proximal articular surface; IZ, interzone; DAS, distal articular surface; DEC, distal epiphyseal cartilage). Statistical analysis was performed on measurements using the paired Student's t-test where * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

It is evident from our studies, that movement and not loading (that is preserved in DMB treated limbs) facilitates an increase in epiphyseal breadth and longitudinal length by engendering volumetric expansion of the limb cartilage components. In contrast, movement fails to produce significant influences upon bone formation, with neither form of immobilization modifying bone's volume or diaphyseal breadth. Thus, it appears that the factors regulating immobilization-induced changes in element length, are not the same as those regulating bone's diaphyseal width, and that cartilage, but not bone, volume is lost with immobilization. This may be interpreted in several ways. The simplest is that embryonic chondrogenesis but not osteogenesis, exhibits sensitivity to extrinsic mechanical stimuli. Another interpretation is that

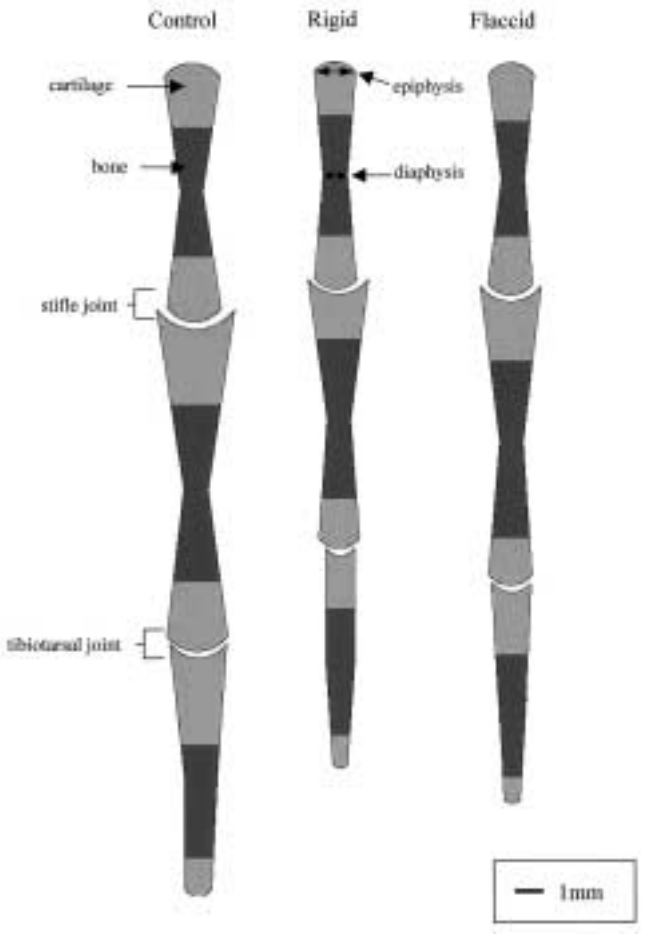

Figure 10: Schematic diagram showing effects of rigid (DMB) and flaccid (PB) paralysis on element length, epiphyseal and diaphyseal breadth and proportional distribution of bone (dark grey) and cartilage (light grey) in developing limbs at stage 36-39 compared to control, Tyrode's solution-treated, chicks.

rates of endochondral bone formation are dramatically increased in response to both flaccid and rigid paralysis, such that bone volume remains relatively unaffected whilst marked diminution in cartilage volume is apparent. The mechanisms regulating these changes are the subject of current investigation.

Interestingly, the immobilization-induced reduction in breadth is most dramatic in the distal epiphyses, with the effects of rigidity being consistently more marked than those of flaccid paralysis. This suggests that both loading and movement contribute to normal increases in epiphyseal breadth; with static loads apparently suppressing and movement promoting such increases. Further, rigid immobilization induced greater reduction in element length, suggesting that movement and loading exert similar influences on such longitudinal expansion. Nonetheless, these pronounced effects evident in the distal elements are consistent with the proximo-distal developmental progression sequence.

Not surprisingly, these neuromuscular agents also have actions beyond the skeletal nervous system. Both decrease heart rate and it has been suggested that in the egg-bound embryo, supplies of oxygen and energy-providing proteins are limiting factors for growth[35]. Therefore, drug-induced decreases in heart rate may reduce the rate of blood flow, which in turn would reduce oxygen delivery, from the air space, and nutrient delivery, from the yolk sac and so decrease growth in paralysed chicks. Alternatively, it is possible that movement facilitates attainment of embryo growth potential.

Thus, our findings may be interpreted as support for the notion that differential growth regulates joint cavity formation. This conclusion differs from that made by Drachmann and Sokoloff[19]. They concluded that the effects of rigid paralysis on joint formation were likely to be independent of growth, as their studies described only slight reductions in bone length and body mass. This difference may be due to the contrasting modes of drug administration used, or might reflect the fact that immobility was induced at earlier stages of development than in our investigations. The timing and term of immobility in our own studies may therefore represent a period of growth and skeletal development that is more dependent on movement.

The development of a novel flaccid form of paralysis may help clarify such complex issues. One important aspect resultant for our establishment of the PB model is its potential reversibility. PB is a non-depolarising competitive antagonist of the transmitter action of acetylcholine, which itself is hydrolysed by acetylcholinesterases[32]. Amongst the possibilities that such acetylcholinesterase-mediated reversibility would allow us to investigate, is whether movement contributes to retaining normal proximo-distal development, and whether movement's influence is incremental or time-dependent, or if the cavitation process is indeed recoverable.

However it is clear that as a result of rigid or flaccid immobilisation the capacity to produce a functional joint cavity is lost. Furthermore, one established associate of cavitation, the formation of a HA-rich ECM component is greatly diminished, confirming that movement is a pre-requisite for such HA enrichment at the presumptive joint. This suggests that in immobilized limbs the primitive, interzonal blastemal cells are capable of adapting their matrix to reflect their new 'immobile' mechanical environment.

In conclusion, the pre-cavitational influence of the 2 distinct forms of short-term immobilization suggests that dynamic components of articulation exert a dominant effect on initial cavity generation. However, their effect upon cavity maintenance differs, with greater loss of normal cavity integrity evident in flaccid limbs. Therefore from our studies it appears that the joint loading, engendered by rigid paralysis, provides partially cavitated joints with some stimulus which acts to retain joint cavity integrity along with high HA levels at this site.

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