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Original Article

Hylonome

Digigait™ quantitation of gait dynamics in rat rheumatoid arthritis model

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Abstract

Introduction. Rheumatoid arthritis (RA) is characterized by joint pain, allodynia and hyperalgesia. The rat carrageenan model utilizes inflammation-associated pain following injection of the knee joint to model RA. Traditional assessment of pain in these models utilizes behavioral scoring or manual measurements, methods that are labor intensive and prone to subjective interpretation. This study utilizes the Digigait™ system to objectively quantify movement and gait dynamics in a monoarthritic rat model. **Material and Methods.** A pilot study in rats selected "natural" runners using Digigait™, and also measured inter and intraday variability as well as effects of anesthesia on gait dynamics. In the main study, 12 female rats were tested at baseline, divided in two groups of 6 rats, briefly anesthetized with isoflurane and injected with 60 µl of 2% lambda carrageenan or vehicle; Digigait testing was repeated 2 and 4 hours post injection and data analyzed. **Results.** Selection of "natural" runners significantly contributed to accuracy and reproducibility of gait parameters obtained by the Digigait™ system. There was minimal intra and inter day variation between individual rats and 4 minutes of isoflurane anesthesia had no effect on gait dynamic at 2 and 4 hours post administration. In the main study a highly reproducible gait signature in the injected limb, and well coordinated adaptation of gait during locomotion in the non-affected limbs were noted as short-term changes following carrageenan injection. **Conclusion.** Digigait™ system was found to be an objective and reliable method for quantification of early behavioral changes consistent with allodynia and hyperalgesia in an inflammatory pain model.

Keywords: Digigait, Acute Inflammatory Pain, Gait Analysis, Carrageenan, Rat

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting the synovial membrane of joints and tendon sheaths characterized by cytokine mediated symmetrical polyarthritis and profound periarticular bone destruction. Clinical signs seen with RA include, joint pain and inflammation but also allodynia and hyperalgesia. RA results in progressive disability due to loss of function in the affected joints caused primarily by abnormalities of cartilage and bone, but also synovial tissue and surrounding muscles and nerves, all of which could independently contribute to gait disturbances, that can progressively worsen with time as disease takes chronic clinical course[1]. The pathogenesis of RA is poorly understood and there is currently no curative therapy. At best symptomatic treatments are available to alleviate the manifestations of RA including severe pain. Aspects of human RA pathophysiology such as pain associated with periarticular destruction can be modeled preclinically by injection of inflammatory agents into the footpad or knee joint of rodents[2,3]. Carrageenan is one such commonly used irritant that elicits an acute, local inflammatory reaction resulting in pain, edema and hyperalgesia, all of which are hallmarks of RA in humans. Unilateral intra-articular injection of carrageenan in rats is a well-described animal model of acute pain[2,3]. Many preclinical models rely on behavioral scoring or manual measurements to quantitate the musculoskeletal consequences of pain. Analysis of this nature requires trained experienced personnel, is time and labor intensive, and prone to subjective data acquisition and interpretation. Recently, novel *in vivo* technologies such as the

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Gait Indices	Description
1. Swing Time (sec)	The forward portion of the stride during which the paw is not in contact with the belt
2. Stance/Swing (ratio)	The ratio of Stance time to Swing time
3. Braking Time (sec)	The time between initial paw contact with the belt to the maximal paw contact
4. Stance Time (sec)	The portion of the stride in which the paw remains in contact with the belt
5. % Stance/Stride	The percent of time that the Stance Time contributes to one complete Stride cycle
6. Stride Length (cm)	The distance between initial contacts of the same paw in one complete stride
7. Stride Time (sec)	The amount of time to complete one complete stride for one limb
8. % Swing/Stride	The percent of time that the Swing Time contributes to one complete Stride cycle
9. % Propulsion/Stride	The percent of time that the Propulsion Time contributes to one complete Stride cycle
10. Paw Area (cm^2)	The maximal paw area in contact with the treadmill during the stance phase of the step cycle
11. Stance Width (cm)	The distance between the two front feet or the two hind feet as measured from the middle of the paw area

Table 1. Description of gait parameters that were used to evaluate change in gait following intraarticular injection of carrageenan.

CatWalk™ and Digigait™ have been developed as non-invasive tools that enable objective assessment of movement and gait dynamics in animal models[4-8].

The main goal of this study was to test the hypothesis that the Digigait™ system can generate reliable objective measurements of movement and gait dynamics in a rat model of RA. As a refinement and prior to the main study we conducted a set of pilots aimed to establish: 1) Selection criteria for enrolling animals that would voluntarily walk on the treadmill component of the device thus ensuring sufficient numbers of animals successfully complete a study, 2) Establish inter and intraday variability within subject and 3) Assess effects of isoflurane anesthesia on gait dynamics.

Material and methods

Animals. The initial population was comprised of twenty male and twenty female Crl:CD rats from Charles River (Fall River, MA). At arrival, rats were 12 weeks old and weighed between 250 and 275 grams. The rats were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited research facility. All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the "Guide for the Care and use of Laboratory Animals". Rats were acclimatized to the vivarium for one week before starting the experiment. All rats were singly-housed in polycarbonate microisolator cages with environmental conditions maintained at 23±1°C, 55±5% humidity, with a 12-hour light/dark cycle; water and standard rat chow *ad libitum*. Body weights were recorded once per week throughout the study.

Gait analysis. Gait analysis was quantified with a Digigait™ Imaging system (Mouse Specifics Inc., Boston, MA) using the method described by Vincelette et al.[7]. In brief a video camera mounted below a transparent treadmill belt captures ventral images of the subject. The images are automatically digitized and software algorithms analyze the digital images to define the area of each paw, and generate a set of periodic waveforms that describe the advance and retreat of the four limbs relative to the treadmill belt through consecutive strides. The software identifies the portions of the paw that are in contact with the treadmill belt in the stance and swing phase of the stride, and measures and calculates numerous postural and kinematic metrics of gait dynamics.

A range of treadmill speeds were tested to determine the maximum rate at which rats would easily walk thereby eliminating variance due to self-selection[4-6]. One rat was placed on the treadmill which was set at 25 cm/sec, a physiologic speed at which rats consistently walked for at least 4 complete strides. Based on published results with Digigait™ or similar systems[4-7], we chose 11 parameters for gait analysis (Table 1).

Study rationale and design. Prior to the main study, a set of pilot studies were conducted in order to establish: 1) Selection criteria for enrolment of subject animals; 2) Determine within subject interday and intraday variability and 3) Assess the effect of isoflurane anesthesia on gait dynamics. Following completion of pilot studies female rats were injected with carrageenan to generate model of RA. This model was used to establish applicability of Digigait™ system to objectively assess gait dynamic 2 and 4 hours following intraartcicular injection of carrageenan.

1) Selection criteria for enrolment of subject animals. The acclimation and selection process involved 20 male and 20 female rats tested in 4 trials using Digigait™ over one week. On day 1 each rat was acclimated to the Digigait chamber with the treadmill off for 30 to 45 seconds after which the treadmill was slowly sped up to 5 cm/sec for additional 1 to 2 seconds. The rat was then removed and put back to its cage. For days 2-5, each animal was acclimated to the Digigait chamber with the treadmill off for 30 to 45 seconds

Female rats (n=12)	Left Front	CV	ICC	Right Front	CV	ICC	Left Hind	CV	ICC	Right Hind	CV	ICC
Parameter	**Foot (LFF)**	**%**		**Foot (RFF)**	**%**		**Foot (LHF)**	**%**		**Foot (RHF)**	**%**	
Swing (sec)	0.15±0.02	5.55	0.32	0.15±0.03	3.63	0.39	0.13±0.02	3.96	0.57	0.12±0.03	2.85	0.57
Stance/Swing (ratio)	2.06±0.32	5.23	0.32	2.02±0.38	5.04	0.26	2.66±0.40	5.16	0.47	2.83±0.40	2.45	0.53
Braking (sec)	0.11±0.04	7.82	0.43	0.16±0.04	7.27	0.23	0.06±0.052	3.38	0.71	0.08±0.04	10.2	0.24
Stance (sec)	0.30±0.03	3.58	0.63	0.30±0.04	3.98	0.37	0.34±0.03	2.07	0.60	0.35±0.03	2.07	0.39
% Stance/Stride	66.92±3.48	1.83	0.35	66.38±4.46	2.10	0.26	72.32±3.11	1.32	0.44	73.62±2.67	0.54	0.50
Stride Length (cm)	11.23±1.09	3.32	0.72	11.34±1.28	2.25	0.45	11.74±1.00	1.78	0.66	11.72±1.02	2.17	0.49
Paw Area (cm^2)	1.07±0.19	7.93	0.25	1.12±0.23	9.21	0.35	2.53±0.38	6.04	0.59	2.56±0.42	6.12	0.46
Stride (sec)	0.45±0.04	3.39	0.72	0.45±0.05	2.30	0.22	0.47±0.04	1.79	0.67	0.47±0.04	2.11	0.51
% Swing/Stride	33.08±3.48	3.71	0.35	33.63±4.46	4.15	0.41	27.68±3.11	3.44	0.44	26.38±2.67	1.49	0.50
% Propulsion/Stride	42.15±8.62	4.75	0.30	41.03±8.05	3.49	0.50	58.92±4.65	2.13	0.49	56.81±6.92	3.27	0.05
Stance Width (cm)	2.19±0.45	3.57	0.27	*	*	*	2.30±0.41	2.83	0.73	*	*	*

Male rats (n=11)	Left Front	CV	ICC	Right Front	CV	ICC	Left Hind	CV	ICC	Right Hind	CV	ICC
Parameter	**Foot (LFF)**	**%**		**Foot (RFF)**	**%**		**Foot (LHF)**	**%**		**Foot (RHF)**	**%**	
Swing (sec)	0.16±0.03	3.63	0.58	0.16±0.03	5.28	0.03	0.13±0.02	0.57	0.05	0.13±0.02	4.99	0.21
Stance/Swing (ratio)	1.94±0.38	4.82	0.56	1.98±0.42	4.93	0.09	2.83±0.42	3.58	0.30	2.90±0.42	5.02	0.48
Braking (sec)	0.14±0.04	13.1	0.19	0.16±0.04	6.81	0.28	0.05±0.02	10.3	0.03	0.05±0.02	14.0	0.14
Stance (sec)	0.30±0.03	2.19	0.15	0.31±0.04	4.88	0.19	0.35±0.04	3.90	0.74	0.35±0.05	4.06	0.72
% Stance/Stride	65.42±4.62	1.81	0.61	65.76±4.98	2.02	0.04	73.64±2.76	1.02	0.31	73.93±3.57	1.49	0.53
Stride Length (cm)	11.55±1.20	1.57	0.06	11.66±1.37	4.28	0.34	12.02±1.19	2.86	0.53	11.97±1.31	3.58	0.46
Paw Area (cm^2)	1.23±0.21	6.05	0.37	1.31±0.26	2.56	0.41	3.02±0.31	5.73	0.17	2.91±0.40	5.80	0.11
Stride (sec)	0.46±0.05	1.57	0.05	0.47±0.06	4.26	0.32	0.48±0.05	2.90	0.52	0.48±0.06	3.59	0.44
% Swing/Stride	34.58±4.62	3.43	0.61	34.25±4.98	3.88	0.04	26.36±2.76	2.86	0.31	26.07±3.57	4.24	0.53
% Propulsion/Stride	34.88±7.50	9.92	0.12	32.25±6.67	8.48	0.02	63.54±5.02	0.61	0.32	62.62±5.18	3.39	0.39
Stance Width (cm)	2.03±0.46	5.04	0.83	*	*	*	2.59±0.33	1.58	0.59	*	*	*

Table 2. show mean ± SD of gait parameters recorded over 5 consecutive days for female (n=12) and male (n=11) rats, % CV data and intra-class correlation (ICC) analysis.

and the treadmill slowly sped up to 25 cm/sec. The treadmill was held at 25 cm/sec for 5 to 10 seconds, images captured and animals subsequently removed. Each rat was scored using the following system:

1= reaches speed (25 cm/sec) with no problems or coaxing*
2= reaches speed on first attempt with minimal intervention
3= reaches speed on second attempt with moderate coaxing
4= reaches speed on more than three attempts with constant coaxing
5= does not run or fails to reach speed

(Coaxing is gentle physical contact using forceps)*

Rats scoring 1 or 2 on at least three consecutive days were chosen for further study, animals scoring 3 or 4 were considered candidates for rescoring with the selection paradigm. Twelve female and 11 male rats (out of original 20) met the criteria for study enrolment and were used to assess variability and the effects of isoflurane on gait dynamics.

2) Interday and intraday variability. Twelve females and 11 male pre-selected rats were tested once per day, Monday through Friday equaling 5 consecutive trials. Recording was done between 13:00 PM and 15:00 PM for each trial. Briefly, each rat was acclimated to the chamber for 30 to 45 seconds before the treadmill was taken up to speed (25 cm/sec). Video images were captured for 5 to 10 seconds of consistent walking/running, the treadmill turned off, and rats returned to their home cages. Rats were scored using the selection criteria described above to track their treadmill compliance. In addition, selected rats were tested 3 times the same day at 13:00 PM, 15:00 PM and 17:00 PM with males and females run on separate days. Each trial was run as described earlier for interday variability.

3) Effect of isoflurane anesthesia on gait dynamics. The same cohort of 12 female and 11 male rats were used in this study. At 13:00 PM selected rats were Digigait™ tested for one trial as described above. Immediately after testing each rat was placed in an IMPAC6® (VetEquip Inc., Pleasanton, CA) anesthesia chamber and exposed to 2.5 % isoflurane in 100% oxy-

	LFF Vehicle		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.17±0.04	0.17±0.03	0.18±0.05
Stance/Swing (ratio)	2.03±0.37	2.00±0.11	1.93±0.68
Braking (sec)	0.14±0.06	0.15±0.05	0.13±0.04
Stance (sec)	0.33±0.04	0.33±0.04	0.31±0.04
% Stance/Stride	66.77±3.60	66.37±1.27	64.32±7.22
Stride Length (cm)	12.53±1.68	12.25±1.47	12.27±1.29
Paw Area (cm^2)	1.01 ±0.20	1.02±0.33	0.96±0.12
Stride (sec)	0.50±0.07	0.49±0.06	0.49±0.05
% Swing/Stride	33.23±3.60	33.63±1.27	35.68±7.22
% Propulsion/Stride	38.80±9.36	35.85±10.38	37.40±13.01
Stance Width (cm)	2.48±0.41	2.32±0.26	2.42±0.23

	LFF Carrageenan		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.20±0.04	0.18±0.01	0.15±0.04
Stance/Swing (ratio)	1.82±0.54	1.92±0.28	2.15±0.40
Braking (sec)	0.17±0.04	0.17±0.04	0.16±0.03
Stance (sec)	0.34±0.04	0.33±0.03	0.32±0.03
% Stance/Stride	63.55±6.71	65.33±3.22	67.68±4.38
Stride Length (cm)	13.35±0.24	12.75±0.72	11.73±1.37[a]
Paw Area (cm^2)	0.97±0.15	1.03±0.16	1.02±0.28
Stride (sec)	0.53±0.01	0.51±0.03	0.47±0.06[a]
% Swing/Stride	36.45±6.71	34.67±3.22	32.32±4.38
% Propulsion/Stride	31.55±9.55	31.77±5.23	34.65±7.23
Stance Width (cm)	2.50±0.49	2.23±0.36	2.05±0.33

Table 3. Gait parameters for the **left front foot** (LFF) of **female** rats. Data were recorded at baseline (time 0) and 2 and 4 hours following injection with carrageenan or vehicle. Data are expressed as mean ± SD; n=6 rats/group. [a]=significantly different from 0 and 2 hours for carrageenan treated rats using paired t-test ($p<0.05$); [b]=significantly different from vehicle treated rats at each time point using student unpaired t-test ($p<0.05$).

gen at a flow rate of 2 L/min. Rats appeared unconscious at 2 minutes and remained in the chamber for 4 minutes total. The rat was then removed from the chamber, allowed to recover in its home cage, and Digigait™ testing repeated at 2 and 4 hours post anesthesia, after rats fully recovered from anesthesia.

Main study - Gait dynamics in rat model of RA. Since results from the pilot studies did not reveal difference in gait dynamic between male and female rats and we had more female rats available for the main study, only female rats were utilized to create acute model of RA. Twelve female rats were baseline Digigait™ tested, randomly divided in two groups of 6 rats and briefly anesthetized with isoflurane as described above. The right knee was surgically prepared and 60 µl of 2% lambda carrageenan (λ carrageenan, type IV, Sigma Chemical Company, St. Louis, Missouri) in saline or vehicle control (saline) injected through the patellar ligament into the knee joint. Rats were then returned to their cages for recovery and Digigait testing was repeated 2 and 4 hours post injection and rats were euthanized after the final trial.

Statistical Methods. The aim in pilot studies was to compare gait measurements in the same subject rats at baseline with subsequent measurements at 2 and 4 hours. The aim in the main study was A) to compare gait measurements in the same subject rats before and after (2 and 4 hours) treatment with vehicle or carrageenan and B) to compare gait parameters in vehicle to carrageenan treated animals at all three time points (0, 2 and 4 hours). Data were analyzed with repeated-measures analysis of variance (ANOVA) to compare gait parameters over time within a group and factorial ANOVA to compare gait parameters between control and carrageenan treated rats. Student's paired *t*-test was used to compare gait parameters obtained in rats within the same study group and

	RFF Vehicle		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.17±0.027	0.16±0.03	0.18±0.05
Stance/Swing (ratio)	1.98±0.46	2.00±0.28	1.93±0.49
Braking (sec)	0.17±0.06	0.17±0.06	0.19±0.02
Stance (sec)	0.32±0.03	0.32±0.04	0.32±0.03
% Stance/Stride	65.80±4.57	66.53±3.19	65.033±6.84
Stride Length (cm)	12.13±0.79	12.03±1.35	12.47±1.41
Paw Area (cm^2)	1.13±0.32	0.96±0.20	1.00±0.11
Stride (sec)	0.49±0.03	0.48±0.05	0.49±0.06
% Swing/Stride	34.20±4.57	33.47±3.19	34.97±6.84
% Propulsion/Stride	31.60±9.36	31.37±11.14	27.27±5.32
Stance Width (cm)	2.48±0.41	2.32±0.26	2.42±0.23

	RFF Carrageenan		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.19±0.02	0.17±0.03	0.13±0.03[a]
Stance/Swing (ratio)	1.93±0.25	2.02±0.31	2.62±0.30[a]
Braking (sec)	0.19±0.05	0.16±0.09	0.11±0.04
Stance (sec)	0.35±0.01	0.34±0.02	0.33±0.03
% Stance/Stride	65.48±3.03	66.47±3.36	71.60±4.41
Stride Length (cm)	13.40±0.61	12.77±0.87	11.67±1.32
Paw Area (cm^2)	1.14±0.27	1.06±0.17	0.95±0.18
Stride (sec)	0.54±0.02	0.51±0.03	0.47±0.04[a]
% Swing/Stride	34.52±3.03	33.53±3.36	28.40±4.41
% Propulsion/Stride	30.58±9.01	34.50±3.12	35.07±5.69[b]
Stance Width (cm)	2.50±0.49	2.23±0.36	2.05±0.33

Table 4. Gait analysis of the **right front foot** (RFF) of female rats. Data were recorded at baseline (time 0) and 2 and 4 hours following injection with carrageenan or vehicle. Data are expressed as mean ± SD; n=6 rats/group. [a]=significantly different from 0 and 2 hours for carrageenan treated rats using paired t-test ($p<0.05$); [b]=significantly different from vehicle treated rats at each time point using unpaired t-test ($p<0.05$).

unpaired *t*-test was used to compare gait parameters between control and carrageenan treated rats. Intra-class correlation coefficient (ICC) was used to estimate the proportion of total variability and reproducibility using "R" program (R-project. Org, Vienna, Austria). All results are presented as the mean ± standard deviation (SD). Differences were considered statistically significant when the *p* value was less than 0.05.

Results

Animal selection. Of the twenty original female rats, 9 were considered as good candidates (fully compliant) and five were considered for retesting. Similarly, of the twenty original males, 7 rats were considered as good candidates (fully compliant) and 5 were considered for retesting. Animals identified as non-compliant on Day 1 rarely showed improvement upon repeat trials. Figure 1 shows the fraction

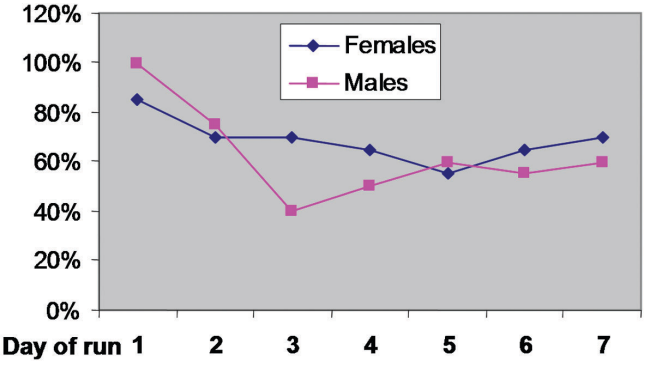

Figure 1. The distribution of rats that selection scored 1 to 3 for three consecutive days.

	LHF Vehicle		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.13±0.02	0.13±0.03	0.14±0.01
Stance/Swing (ratio)	2.78±0.33	2.88±0.71	2.83±0.24
Braking (sec)	0.04±0.02	0.05±0.03	0.04±0.02
Stance (sec)	0.36±0.04	0.37±0.04	0.38±0.04
% Stance/Stride	73.48±2.43	73.78±4.05	73.75±1.80
Stride Length (cm)	12.23±1.16	12.53±1.31	12.92±1.21
Paw Area (cm^2)	2.25±0.25	2.18±0.62	2.47±0.25
Stride (sec)	0.49±0.05	0.51±0.05	0.52±0.05
% Swing/Stride	26.52±2.43	26.22±4.05	26.25±1.80
% Propulsion/Stride	64.27±4.03	63.03±7.79	65.40±3.67
Stance Width (cm)	2.65±0.36	2.68±0.51	2.65±0.38

	LHF Carrageenan		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.14±0.01	0.14±0.01	0.12±0.02
Stance/Swing (ratio)	2.87±0.23	2.72±0.26	3.62±0.72[a,b]
Braking (sec)	0.05±0.01	0.06±0.03	0.04±0.02
Stance (sec)	0.40±0.03	0.38±0.03	0.42±0.05
% Stance/Stride	73.97±1.72	73.02±1.62	77.82±2.47[a,b]
Stride Length (cm)	13.37±0.67	12.98±1.01	13.48±1.19
Paw Area (cm^2)	2.55±0.21	2.61±0.06	2.78±0.40
Stride (sec)	0.54±0.03	0.52±0.04	0.54±0.05
% Swing/Stride	26.03±1.72	26.98±1.62	22.18±3.47[a,b]
% Propulsion/Stride	65.07±3.18	61.37±8.15	70.40±3.11[a]
Stance Width (cm)	2.40±0.34	2.20±0.49	2.38±0.29

Table 5. Gait analysis of the **left hind foot** (LHF) of the **female** rats. Data were recorded at baseline (time 0 point) and 2 and 4 hours following injection with carrageenan or vehicle. Data are expressed as mean ± SD; n=6 rats/group. [a]=significantly different from 0 and 2 hours for carrageenan treated rats using paired t-test ($p<0.05$); [b]=significantly different from vehicle treated rats at each time point using unpaired t-test ($p<0.05$).

of rats from the initial population that selection scored 1 to 3 for three consecutive days. For animals rated 1-3 attrition for both males and females was approximately 40% by the third day of testing and most rats performed consistently from day 3 onward.

Variability. There were no statistically significant difference in gait parameters between various time points in both inter- and intraday variability studies using paired t-test. All gait indices measured under conditions described in this manuscript yielded reproducible results over 1 week in both male and female rats. Example of interday variability of gait parameters showing mean ± SD of five measurements, CV% and ICC data is presented in Table 2. The coefficient of variation for day to day and intra day measurements (data not shown) suggest that under our conditions Digigait™ is capable of generating reproducible results of gait parameters.

Effect of isoflurane anesthesia on gait dynamics. The carrageenan model requires the use of brief anesthesia for intra-articular injections. To assess the effect of anesthesia on gait dynamics rats were Digigait tested before and after brief isoflurane anesthesia. When compared to pre-anesthesia values there were no significant differences in gait parameters 2 and 4 hours post isoflurane anesthesia (data not shown).

Effect of intra-articular injection of carrageenan on gait dynamics in female rats

The effects of carrageenan injection on left front foot (LFF) gait dynamics are presented in Table 3. When compared to T_0, carrageenan injected rats exhibited shorter Stride ($p=.0402$) and Stride length ($p=.0473$) 4 hours post injection. A trend towards shorter Swing, Stance, %

	RHF Vehicle		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.14±0.02	0.14±0.02	0.14±0.01
Stance/Swing (ratio)	2.58±0.19	2.73±0.23	2.70±0.32
Braking (sec)	0.06±0.03	0.08±0.02	0.05±0.03
Stance (sec)	0.36±0.04	0.37±0.04	0.36±0.04
% Stance/Stride	72.12±1.39	73.13±1.78	72.80±2.26
Stride Length (cm)	12.53±1.41	12.60±1.37	12.45±1.21
Paw Area (cm^2)	2.38±0.31	2.08±0.67	2.24±0.30
Stride (sec)	0.50±0.06	0.50±0.05	0.50±0.05
% Swing/Stride	27.88±1.39	26.87±1.78	27.20±2.26
% Propulsion/Stride	60.38±6.81	57.08±3.98	61.78±6.08
Stance Width (cm)	2.65±0.36	2.68±0.51	2.65±0.38

	RHF Carrageenan		
Parameter	**Time 0**	**Time 2**	**Time 4**
Swing (sec)	0.14±0.01	0.14±0.02	0.17±0.02[a,b]
Stance/Swing (ratio)	2.77±0.24	2.70±0.35	1.80±0.69[a,b]
Braking (sec)	0.05±0.02	0.07±0.02	0.07±0.02
Stance (sec)	0.39±0.01	0.38±0.03	0.29±0.07[a]
% Stance/Stride	73.33±1.70	72.67±2.64	62.27±9.63[a,b]
Stride Length (cm)	13.42±0.34	13.07±1.06	11.65±2.05[a]
Paw Area (cm^2)	2.57±0.26	2.49±0.23	1.63±0.60[a,b]
Stride (sec)	0.54±0.01	0.52±0.04	0.47±0.08[a]
% Swing/Stride	26.67±1.70	27.33±2.64	37.73±9.63[a,b]
% Propulsion/Stride	63.78±2.37	59.58±5.11	47.47±12.9[a,b]
Stance Width (cm)	2.40±0.34	2.20±0.49	2.38±0.29

Table 6. Gait analysis of the **right hind foot** (RHF) of the **female** rats. Data were recorded at baseline (time 0 point) and 2 and 4 hours following injection with carrageenan or vehicle. Data are expressed as mean ± SD; n=6 rats/group. [a]=significantly different from 0 and 2 hours for carrageenan treated rats using paired t-test ($p<0.05$); [b]=significantly different from vehicle treated rats at each time point using unpaired t-test ($p<0.05$).

Swing/Stride, Stance width and % Propulsion/stride was also noted, but results did not reach statistical significance. Also, there was trend toward increasing Stance/Swing ratio, % Stance/Stride and Paw area, although those changes did not reach statistical significance.

Table 4 shows the results of right front limb (RFL) gait analysis. Rats injected with carrageenan exhibited significant increase in Stance/Swing ratio (p=.0433) and decreased Swing (p=.0361) and Stride (p=.0416) 4 hours post injection relative to time 0. There was also trend toward increasing % Stance/Stride and % Propulsion/Stride parameters, and decrease in Breaking, Stance, Stride, Paw area and Stride length; however noted differences were not statistically significant. Also, carrageenan injected rats showed higher values for % propulsion/Stride (p=.0341) compared to vehicle controls at 4 hour time point.

The results of left hind foot (LHF) gait analysis are presented in Table 5. Female rats injected with carrageenan exhibited significantly increased Stance/Swing Ratio at 4 hour time point relative to carrageenan group T_0 (p=.0301) but also to vehicle treated rats (p=.0305). Similarly % Stance/Stride was increased in carrageenan group at T_4 relative to carrageenan T_0 (p=.0290) and relative to vehicle controls (p=.0236). % Propulsion/Stride was higher in carrageenan rats at 4 hour time point relative to T_0 (p=.0403) while % Swing/Stride parameter in carrageenan treated rats at 4 hours post injection showed significantly lower values compared to T_0 in for the same group of rats (p=.0290) but also relative to vehicle controls (p=.0293).

As can been seen in Table 6 most gait parameters were affected in the carrageenan injected right hind foot (RHF). Female rats injected with carrageenan exhibited significantly

increased or decreased the following parameters relative to T_0 in the same group of rats: Swing (p=.0223), Stance/Swing ratio (p=.0120), Stance (p=.0366), % Stance/Stride (p=.0215), Stride length (p=.0392), Paw area (p=.0466), Stride (p=.0486), %Swing/Stride ratio (p=.0264) and % Propulsion/Stride (p=.0247). Respective significant changes in Swing (p=.0223), % Swing/Stride (p=.0261), Stance/Swing ratio (p=.0154), % Stance/Stride (p=.0261), Paw area (p=.0498) and % Propulsion/Stride (p=.0338) were also seen between carrageenan treated rats at the 4 hour time point and all time points for vehicle treated rats.

Discussion

Our previous experience indicates that up to 50% of rats will not run on a moving treadmill and that drop-outs may negatively impact study integrity. To avoid losing animals and to ensure that the normal affinity of rats for running would not bias the results, we established quick and reliable criteria to pre-select animals for study enrollment. By identifying "natural" runners during the selection phase of our study, we were able to complete all experiments using the same subset of rats without losing animals due to poor compliance or injury. In addition we mitigated the propensity for rats to run at self-selected speeds as a confounding factor; all rats enrolled adopted the treadmill speed we set. During the selection process we found that rats which resisted or were poor runners (scores 4-5) in the first 3-4 days didn't improve they ability to run on the treadmill over time. Once "natural" runners were identified (scores 1-2), they consistently performed well throughout the entire 4 weeks of the study. We found that approximately 40 % of both male and female CD rats will not voluntarily cooperate in running based experiments. Scientist should take this into account when planning *in vivo* experiments utilizing Digigait™ or similar technologies.

As evidenced by the relative standard deviation (RSD) gait parameters obtained by the Digigait™ system yield highly reproducible results in male and female CD rats. Both inter and intra day variation was almost identical. Since our selection process likely made a significant contribution to the low RSD we conclude that the Digigait™ system is capable of producing highly accurate data when used with an appropriate study population.

It is often necessary to briefly anesthetize animals in order to perform a small surgical procedure, administer a treatment or to collect blood and isoflurane gas anesthesia is commonly used. In our case brief anesthesia was required for intraarticular carageenan injection. To our knowledge, currently there is no published information describing the influence of isoflurane anesthesia on rat gait dynamics. We clearly demonstrated that brief (4 minute) isoflurane anesthesia has no effect on gait parameters in CD rats 2 and 4 hours after recovery and thus was not a biasing factor in this study.

Rodent models of carrageenan-induced local inflammation are commonly used in preclinical setup to evaluate efficacy of anti-inflammatory drugs as well as drugs aimed to alleviate

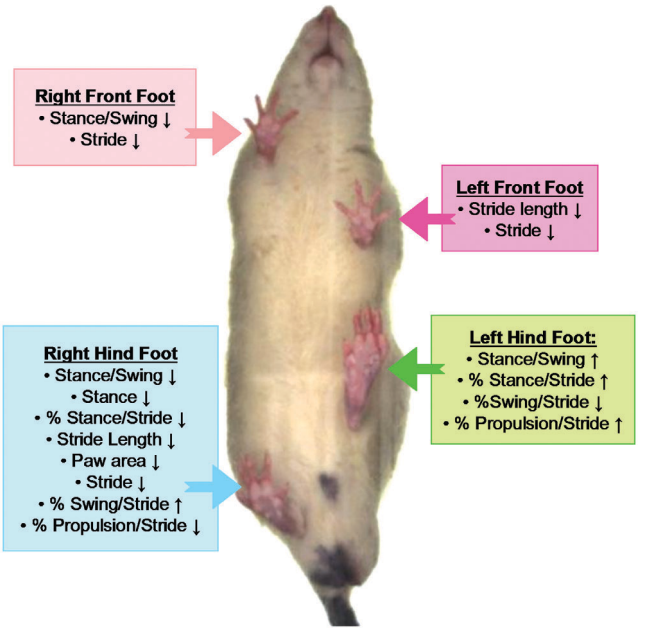

Figure 2. depicts gait parameters that changed 4 hours post intra-articular injection of carrageenan in the right knee of female rats.

inflammation associated pain; hence, the cellular and molecular mechanisms of carrageenan-induced edema are well described in the literature[8-11]. Tissue inflammation initiates the rapid development of clinical pain characterized by both pain from non-noxious stimuli (allodynia) and enhanced pain (hyperalgesia) from noxious stimuli. Evaluation of pain in rat inflammatory models is routinely assessed by a number of behavioral tests including: weight bearing by the affected foot, paw elevation time and paw withdrawal latency[18-22]. In monoarthritic rat models, pain causes restricted movements of the affected knee that is usually assessed by measuring the ankle bend score[23] or struggle threshold score that measures extension angle as an index of pain[24]. Similar to observations obtained in rats, patients with arthritis of the knee joint experience substantial pain following weight bearing or mild mechanical stimulation of the inflamed knee caused by movement or applied pressure[25].

Using the Digigait™ system we sought to objectively identify gait patterns which could serve as reliable markers of acute pain. We employed the carageenan monoarthritic rat model, a well established paradigm of rheumatoid arthritis and associated pain. In monoarthritic rat model of RA we observed significant differences in gait parameters very early after carageenan injection (4 hours post injection) and long before knee swelling or physical deformities occur (summarized in Figure 2). As expected, rats showed the most dramatic changes in gait parameters in the carrageenan injected right hind limb in which, both temporal and spatial gait parameters showed significant changes in response to acute pain. Reduction in Stance, Stride, Paw Area, and derived parameters percent Stance/Stride and Propulsion/Stride were signifi-

cantly different from individual baseline values (T_0) or vehicle controls at T_4. Compensatory gait changes of increased percent Stance/Stride, Swing/Stride, Propulsion/Stride, and Stance/Swing ratio were noted in the contralateral hind limb. Significant but less extensive gait changes were noted in both front limbs comprised of altered stride pattern[26,27].

Gait analysis has been shown to correlate well with classical methods used to assess nociception such as von Frey test[6,22]. Our work is consistent with that of Coulthard et al. who postulated that gait analysis may be an objective method for measuring early behavioral change associated with induced chronic inflammatory pain[28,29]. Results from their study also showed that injection of carrageenan results in reduced paw pressure during walking, and that this symptom represents one of commonly seen signs of mechanical allodynia. Similarly, duration of stance and swing phase were decreased and increased, respectively, indicating compensatory mechanisms used by quadrupeds to alleviate pain arising from the affected limb.

Scientists using classical nociceptive testing methods face several difficulties which may significantly affect study results. In particular tedious data collection, subjective recording or interpretation of responses and dependence on test operator skill and experience. Conversely, the Digigait™ technology automatically records objective measurements, requires minimal engagement of personnel and rapidly captures numerous gait parameters simultaneously in all 4 limbs. Thus, the Digigait™ system allows for complete analysis of animal locomotion including compensatory mechanisms that naturally occur in non-affected limbs. With further study automated gait analysis may allow for defining highly reproducible pain or disease characteristic "gait signatures". The precision of the Digigait™ system and the good correlation between gait analysis and classic nociceptive tests indicates that automated gait analysis technology may also benefit research and animal welfare by allowing scientists to reduce animal numbers required to obtain significant results and possibly replace painful or controversial procedures[30]. Although results from this experiment are encouraging, studies of longer duration will be necessary, in particular if direct comparison of data obtained by Digigait™ and classical methods can be achieved in the same study subjects. In addition, there seems to be substantial difference between individual parameters of gait, with some being very steady, yet sensitive enough to detect early change induced by carrageenan, whereas other parameters appeared to be less reliable due to larger individual and group variability. Therefore, special caution should be applied when interpreting data obtained by the Digigait™ or similar systems, since all gait parameters are not equal and do not have the same power and sensitivity to accurately describe complex changes that occur in arthritic joints. Consequently, "signature" patterns seem to have higher diagnostic value and if established should be carefully implemented along with dominant single parameters of gait in order to best describe gait change for a particular animal model deployed.

Obviously, there are numerous approaches on how to model and assess pain in preclinical studies and choice of animal model, treatment modalities and time point selection for assessment of pain in those models could greatly influence the data. In our study the Digigait™ system produced reproducible changes in gait parameters in the injected limb and potentially adaptive changes in the non-affected limbs at 2 and 4 hours following intra-articular injection of carrageenan. Although one should generally be cautious when extrapolating animal experimental findings to humans, this study suggests that gait parameters are a suitable and objective method for assessment of early behavioral changes associated with inflammatory pain and may reduce the need for classic indices of hyperalgesia and allodinya. To fully evaluate the potential and translational value of emerging non-invasive technologies aimed to objectively assess gait dynamics more research needs to be conducted using different preclinical models of acute and chronic pain. Nevertheless, preselection of "natural runners" prior to study conduct helps minimize attrition and ensures data integrity.

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