Interference of age and repetition of the same noxious stimulus on hyperalgesia[1]

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ABSTRACT.- Ibañez J.F., Posso I.P. & Wallace V. 2010. **Interference of age and repetition of the same noxious stimulus on hyperalgesia.** *Pesquisa Veterinária Brasileira 30(9):777-782.* Departamento de Patologia Geral, Curso de Medicina Veterinária, Universidade Estadual do Norte do Paraná, Campus Luiz Meneghel, Rodovia BR 369 Km 54, Bandeirantes, PR 86360-000, Brazil. E-mail: ibanez@uenp.edu.br

Pain in animals has been recognized for less than one century. Several authors confirm that animals are capable to process, register and modulate nociceptive stimuli in a very similar way to human kind and there are several evidences registering the impact of pain sensation over vital systems interfering on disease outcome. Nevertheless, despite some evidences that animals, as human beings, can store information from past painful experiences less is known about how this so called pain memory works. The aims of this study were: to evaluate if the response to a painful stimuli differs during different stages of life and if repetition of a same acute stimuli in the same animal interferes with expression of hyperalgesia. Thus, 60 rats were selected and arranged in 3 equal groups: 3 months, 6 months, and 9 months of age. All animals were injected 5% formalin solution in the plantar face of hind paw under volatile general anesthesia. Von Frey filaments were applied at 1h, 24h and 48h after sensitization. Injection was repeated twice with a 30-day interval, each time in a different hind paw. Results showed that younger rats express lower hyperalgesia thresholds in the first stimulation compared to elder animals and that repetition of same stimulus diminishes hyperalgesia thresholds when it begins during infant period and augments hyperalgesia thresholds when it begins during elder ages.

INDEX TERMS: Rat, age, development, memory, allodynia, formalin test, algometer.

RESUMO.- [**Interferência da idade e repetição do mesmo estímulo doloroso na hiperalgesia.**] A dor nos animais tem sido reconhecida há pouco manos de um século. Vários autores reconhecem que os animais são capazes de processar, registrar e modular estímulos nociceptivos de modo muito similar aos seres humanos e há várias evidências registrando o impacto da sensação dolorosa sobre os sistemas vitais e curso da doença. Entretanto, a despeito das evidências de que os animais, como os seres humanos, podem armazenar informações passadas de experiências dolorosas pouco se sabe sobre como a chamada memoria de dor funciona. Os objetivos deste estudo foram: avaliar se a resposta a um estímulo doloroso difere em diferentes fases da vida e se a repetição de um mesmo estímulo doloroso agudo no mesmo animal interfere na expressão da hiperalgesia. Assim, 60 ratos foram selecionados e agrupados em três grupos iguais: 3 meses, 6 meses e 9 meses. Foi injetada solução de formalina 5% na face plantar de todos os animais durante anesthesia. O limiar de hiperalgesia foi testado pelo método de filamentos de Von Frey à 1h, 24h e 48h após a sensibilização. A injecão foi repetida duas vezes com intervalo de 30 dias, uma vez em cada membro. Os resultados demosntraram que animais mais jovens possuem limiares menores de hiperalgesia na primeira estimulação, comparados com os mais velhos e que a repetição de um mesmo estímulo doloroso agudo diminui o limiar de hiperalgesia quando o

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primeiro estímulo ocorre nas idades mais tenras e aumenta o limiar quando se inicia em idades mais avançadas.

TERMOS DE INDEXAÇÃO: Rato, idade, desenvolvimento, memória, alodinia, teste da formalina, algiometria.

INTRODUCTION

Several procedures in neonates and in veterinary practice are performed without any pain relief, due to the belief that nervous system is not completely mature right after birth. However, researches proved that newborns of several species are capable to identify noxious stimuli and that they interfere in maturation of sensitive pathways during central nervous system development (Ruda et al. 2000, Johnston & Walker 2003). Apparently this occurs because during childhood central nervous system is prone to activity dependent modulation (Homaister et al. 2009, Walker et al. 2009).

Nociceptive stimulus can evoke major alterations in medullar function, including sensitization. Recently, several authors have purposed detailed theories of how nociceptive stimuli modify central nervous system (Zelter et al. 1997, Al-Chaer et al. 2000, Ruda et al. 2000, Bhutta et al. 2001, Melzak et al. 2001). Although it's known that precocious stimuli modulates sensitive pathways and response to pain later, the amount of injured tissue necessary to modulate such alterations is to discuss (Walker et al. 2009).

Not only after birth, but later during infancy, stimuli can alter neuronal perception of pain. In a study evaluating pain threshold in children aging from 9 to 16 years old and burned from 6 to 24 months of age, authors could confirm that they had lower thresholds compared to children that had never burned their selves (Wollgarten-Hadamk et al. 2009).

Perception of pain is complex and includes psychological and sensorial information that are liable to psychological factors. Children of different ages may experience the same painful stimulus, however, due to differences on development of the ability to graduate and report stimuli or other pain components, responses may vary largely (Goodenough et al. 1999, Al-Chaer et al. 2000). Some authors postulate that children and adults may see pain as a stress and functional disability generator. These stimuli applied frequently and intensively during development may lead to a belief that pain is threatening and this may trigger a defense response and lower the ability to cope with it (Hohmeister et al. 2009).

Postoperatory pain simulation in laboratory animals include, among others, plantar incision, developed by Brennan et al (1996), and formalin test, presented by Dubuisson & Dennis (1977). Formalin tests have been used to simulate postoperatory inflammatory component and presents two phases: phase I with peak at 5 minutes mediated by local sensitive fibers; and phase II, about 15 minutes after injection, mediated by inflammatory mediators and non noxious sensitive fibers (Bhutta et al. 2001, Hogan 2002, Ashmawi et al 2003).

Quantifying hyperalgesia evolves behavioral patterns and devices capable to translate them into numbers. One of these is the von Frey filament set, largely used to measure hyperalgesia (Brenan et al. 1996, Hogan 2002, Ririe et al. 2004).

Recognize, qualify and quantify pain in animals is of essential importance to provide them welfare and in consequence, to humans, once the similarity between sensitive pathways and nociceptive responses in both life kinds (Hogan 2002). It's well known that children submitted to painful stimuli during childhood express less coping ability and tolerance to pain stimuli latter in life (Hohmeister et al. 2009).

An important point is to evaluate the importance of considering the age of animals undergoing pain research protocols, since it may alter behavior and pain thresholds depending on the life stage experiments are proceeded (Al-Chaer 2000, Ririe et al. 2004).

Thus, the aims of this study were to investigate if age interferes in the expression of hyperalgesia after a noxious stimulation, and if repetition of the same noxious stimulus in the same individual interferes on expression of hyperalgesia.

MATERIALS AND METHODS

Sixty Wistar male rats with light period control and *ad libitum* water and food were used. Experimental protocol was approved by Research Ethical Analysis Committee of Hospital das Clínicas and Medicine School, Universidade de São Paulo, on April 25, 2002, under protocol number 246/02.

Animals were divided into three groups (n=20): 3 months (GF3), 6 months (GF6) and 9 months of age (GF9).

Sensitization was gained by administering 0.05ml of 5% formalin solution with an insulin syringe and a 20x5.5G needle in the subcutaneous plantar face of hind paw (Fig.1).

To perform injection animals were anesthetized with halothane[5] in 100% oxygen. After injection animals were identified and devolved to their respective boxes.

Withadraw threshold was measured with von Frey filaments[6] as suggested by Brennan et al (1996). Each filament, in a crescent order was applied three consecutive times with a 3 to 5 seconds interval and if no response was observed, another filament was tested. Positive response was considered when tested animal lifted sensitized paw. When animals' paws were

Fig.1. Hind paw with needle, pointing the site of injection.

[5] Fluothane™, Astra Zeneca do Brasil Ltda, Rodovia Raposo Tavares, Km 26.9, Cotia, São Paulo, SP 06707-000.

[6] Touch Test Sensory Evaluator Kit™, Stoelting, 620 Wheat Lane, Wood Dale, Illinois 60191, USA.

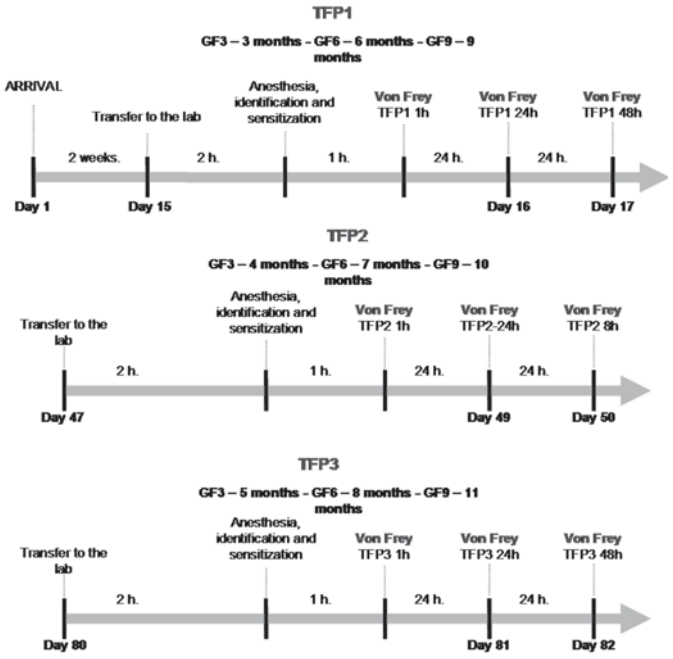

Fig.2. Experimental design illustrating the moments of sensitization and pain tests. TFP, Plantar formalin test (injection of).

already lifted before testing, value attributed was zero, meaning that it was already in pain.

To perform von Frey test, animals were put in a translucid plastic box with a wire mesh floor through which filaments were applied by indirect vision with a mirror.

Sensitization was performed three times (T1, T2 and T3), with a 30 day interval in each group, so that GF3 was tested at 3, 4 and 5 months old; GF6 at 6, 7 and 8 months old and GF9 at 9, 10 and 11 months old. Hyperalgesia was measured at 1h, 24h and 48 after each sensitization (Fig.2).

Kruskall-Wallis test was applied to compare data among groups, repetition and moment. In order to verify which groups showed different means, Mann-Whitney test was performed.

To compare means of data among repetitions during different moments and groups we used Friedman test, repeated to each data set (each repetition).

Global 5% significance level vas controlled with Bonferroni method.

RESULTS

After first sensitization, 100% of GF9 animals presented limb lameness 1h after stimulus against 85% of animals in GF3 and 35% of animals in GF6. Limb lameness was observed in 100% of animals 1h after second and third sensitization.

GF3, GF6 and GF9 had different thresholds in moments T1 - 1h; T1 - 24h; T1 - 48h, T2 - 48h; T3 - 24h, and T3 - 48h (Table 1).

Mann-Whitney analysis revealed that except during T1-24h when GF3 presented higher thresholds, in all other moments of T1 and all moments of T2 and T3, animals in GF3 presented lower thresholds compared to GF6 and GF9 although sometimes differences were numerical, with no statistical significance (Table 2, Fig.3).

Group GF3 showed higher thresholds in T1-24 compared to T3-24h, and although not statistically significant, T2-24h showed intermediary values between T1-24h and T3-24h, suggesting a decreasing pattern in hyperalgesia threshold along repetition of stimuli for GF3 group 24h after sensitization. Group GF9 behaved in an inverse mode, with

Table 1. Descriptive levels (alpha) for Kruskal-Wallis analysis of T1, T2, T3 (p = 0.05). 2004

Test	Moment	p value
T1	1h	0.001
	24h	0.001
	48h	0.001
T2	1h	x*
	24h	0.402
	48h	0.020
T3	1h	x*
	24h	0.008
	48h	0.001

* Zero values.

Table 2. Descriptive levels (alpha) to Mann-Whitney test comparing hiperalgesia among GF3, GF6 and GF9. Means ± standard errors (p=0.05)

Test	Moment	Values von Frey filaments (mN)		Descriptive level (alpha)
T1	1h	GF3-2,6±1,5 mN	GF6-17,5±6,6 mN	0.003
		GF3-2,6±1,5 mN	GF9-0,0 mN	-x-*
		GF6-17,5±6,6 mN	GF9-0,0 mN	-x-*
	24h	GF3-159±27,9 mN	GF6-35,6±9,3 mN	0.001
		GF3-159±27,9 mN	GF9-31,2±3,4 mN	0.002
		GF6-35,6±9,3 mN	GF9-31,2±3,4 mN	0.190
	48h	GF3-25,2±4,7 mN	GF6-63,1±13,9 mN	0.001
		GF3-25,2±4,7 mN	GF9-70,6±5,4 mN	< 0.001
		GF6-63,1±13,9 mN	GF9-70,6±5,4 mN	0.063
T2	48h	GF3-51,7±8,6 mN	GF6-75,3±9,4 mN	0.029
		GF3-51,7±8,6 mN	GF9-132,1±25,9 mN	0.012
		GF6-75,3±9,4 mN	GF9-132,1±25,9 mN	0.426
T3	24h	GF3-33,3±5,8 mN	GF6-65,3±19,7 mN	0.210
		GF3-33,3±5,8 mN	GF9-91,3±20,9 mN	0.002
		GF6-65,3±19,7 mN	GF9-91,3±20,9 mN	0.087
	48h	GF3-29,6±3,2 mN	GF6-124,5±28,6 mN	0.009
		GF3-29,6±3,2 mN	GF9-128,9±25,9 mN	< 0.001
		GF6-124,5±28,6 mN	GF9-128,9±25,9 mN	0.421

* Values equal zero. Values expressed by mean ± pattern error. Bold values (p<0.05)

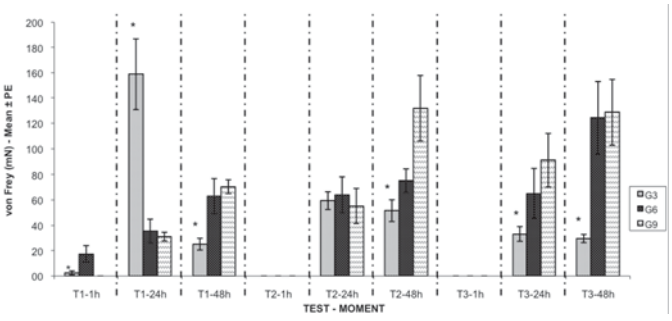

Fig.3. Comparison of means ± standard errors of force eliciting hyperalgesia with von Frey test. Means ± standard errors; * p<0.0167. G3=GF3, G6=GF6, and G9=GF9.

Table 3. Descriptive levels for Friedman test comparing hiperalgesia thresholds among repetition (TFP1, TFP2 e TFP3) in different groups and moments. Means ± standard errors (p=0.05). 2004

Moment	Group	T1	T2	T3	Descriptive level (alpha)
1h	GF3	2,6 ± 1,5 mN	0,0	0,0	0.500
	GF6	17,5 ± 6,6 mN †	0,0	0,0	< 0.001
	GF9	0,0	0,0	0,0	x*
24h	GF3	159,0 ± 27,9 mN †	59,6 ± 7,0 mN	33,3 ± 5,8 mN †	0.006
	GF6	35,6 ± 9,3 mN	64,2 ± 14,2 mN	65,3 ± 19,7 mN	0.054
	GF9	31,2 ± 3,4 mN †	55,4 ± 13,9 mN	91,3 ± 20,9 mN †	0.008
48h	GF3	25,2 ± 4,7 mN †	51,7 ± 8,6 mN †	29,6 ± 3,2 mN	0.016
	GF6	63,1 ± 13,9 mN	75,3 ± 9,4 mN	124,5 ± 28,6 mN	0.776
	GF9	70,6 ± 5,4 mN	132,1 ± 25,9 mN	128,9 ± 25,9 mN	0.534

* Zero. † statistically significant difference (p<0.05).

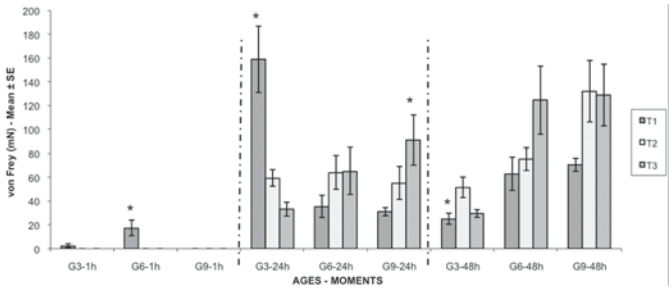

Fig.4. Comparison of means ± standard errors of hiperalgesia threshold among repetitions at 1h, 24h and 48h after sensitization in groups GF3, GF6, and GF9. *p<0.0167. G3=GF3, G6=GF6, and G9=GF9.

T1-24h presenting statistically inferior values than obtained in T3-24h and T2-24h with values numerically intermediary between T1-24h and T3-24h, suggesting an increase in hyperalgesia threshold along repetition of stimuli to GF9 group.

Group GF6 did not show statistical differences among T1-24h, T2-24h and T3-24h, nevertheless, T1-24h, T2-24h and T3-24h followed the same crescent pattern observed in GF9

The same observations were seen in groups GF6 and GF9 in moments T1-48h, T2-48h and T3-48h, when data presented the same crescent pattern, without statistical differences however. A higher hyperalgesia threshold was observed in group GF3 in T2-48h compared to T1-48h and lowering to values similar to T1-48h in moment T3-48h, always with no statistical significance (Table 3, Fig.4).

DISCUSSION

Once personal report is not a reliable option with very young children and animals, pain treatment tends to occur on what is expected. However, if subject doesn't act as expected, behavior can be misinterpreted as non painful. It may be difficult to differ pain from anxiety when stress behavior is present. When submitted to acute pain, either stress or pain are combined and exteriorized by behavior (Zeltzer et al. 1997, Hogan 2002). This could explain the lower hyperalgesia thresholds observed in GF3 group 1 hour after sensitizations, compared to GF6 and GF9.

Stimuli pattern and intensity, as well as time interval and duration of discharges on afferent fibers are imperative to hyperalgesia induction and long time synaptic alterations. Nevertheless, frequency used to evoke these kinds of responses in experimental models is infrequent in nature (Sandküler & Liu 1998, Ririe et al. 2004, Hohmeister et al. 2009).

A large number of hiperalgesia models are based on inflammatory pain produced by inflammatory mediators and peripheral nervous cells in the lesion area, that sensitize primary afferent fibers diminishing depolarization threshold and producing alodinia and hyperalgesia (McKenna & Melzack 2001, Ashmawi et al 2003, Johnston & Walker 2003). The amount of damaged tissue necessary to result in nervous long lasting pathways modification is controverse (Camozzato et al. 2009, Walker et al. 2009). Less intense stimuli may lead to long term activation of wide range neurons even during physiological conditions and this may be an induced afferent hyperalgesia mechanism and could justify the decrease in hyperalgesia threshold in group GF3 during subsequent sensitizations compared to previous ones (Rygh et al. 1999, Johnston & Walker 2003).

It seems that phase I of formalin test occurs by direct nociceptor stimulation, mainly C fibers, and mediators involved in each of two phases are also different: P substance in phase I and histamine, serotonin, prostaglandins and bradikinine during phase II. Phase II depends on dorsal horn stimulation during phase I. It's believed that histamine may facilitate afferent medullar pathways (Ashmawi et al. 2003)

In the present study sensitivity differences were observed among the age groups, with GF3 showing lower hyperalgesia thresholds than GF6 or GF9, which accords to Grunnau et al. (2001) and Johnston & Walker (2003) who postulated that the way stimuli are processed after first occurrence during immature periods of nervous system, may be different, inducing hyperalgesia. Hohmeister et al. (2009) also postulate that infant can barely deal with unpredicted or suddenly events.

Viganó et al. (1998), affirm that mechanical sensitivity thresholds augment with age, however their studies were performed in non previously sensitized individuals. Nevertheless, animals from group GF9 were the only ones showing limb impotence 1h after sensitization and rats from group GF6 presented lower thresholds than GF3, disa-

greeing with the referred authors, but in accordance to Turk et al. (1995) who affirm that the elder individuals are more sensitive to pain.

In a study comparing needle puncture sensitivity in children aging from 7 to 11 years, and 12 to 16 years, younger ones presented lower thresholds than elder ones (Goodenough et al. 1999). In an other study with children undergoing venipuncture and divided in groups that received previous anesthetic block, placebo and no kind of prevention, authors showed age difference with younger children more sensitive, and a strong positive correlation between anxiety and high predictive pain scores (Goodenough et al. 1997).

Pain may not always be related to intensity of trauma or disease, but it must be related also to environmental, emotional and evolutive factors, as previous experiences and the way parents contribute to interpretation of painful stimuli and resulting stress (Gibson 2004, Hohmeister et al. 2009).

Endogenous antinociceptive mechanisms may be activated by environmental stimuli such as battle fields, races, sexual acting or even by individual motivation when adapted do some condition (Sudhakar & Venkatesh 2003, Camozzato et al. 2009, Walker et al. 2009). Perhaps, inexperience to handling and the unknown conditions of the first stimulation may have evoked these antinociceptive mechanisms in GF3 animals, increasing their pain threshold as observed in T1-1h and T1-24h and in T-1h for GF6 ones. Emotions such as fear and anxiety may interfere on pain intensity and quality. Stress induced analgesia may also be observed in large trauma victims. In rats it has been found that c-fos expression diminishes when a painful stimulus is applied during stressful conditions (Magalhães 2003)

There are some suppositions that repeated stimuli in the same place may develop altered disseminated responses during pain perception (Oberlander et al. 2000, Gibson 2004, Camozzatto et al. 2009).

Prolonged exposition to some stimulus models show that some kinds of injury lead to permanent structural and functional alterations, besides to central nervous system reorganizing with neural connection altered patterns (Grunau 1998, Capone & Aloisi 2004).

In our findings, GF3 animals presented lowering thresholds when stimuli was repeated, mainly 24h after sensitization; and GF6 and GF9 animals showed an inverse behavior, with increasing thresholds during repetition of stimulus. Perhaps there is more susceptibility of sensitive changes depending to acute stimuli as the ones produced by formalin in infant ages.

Sandkuler & Liu (1998) affirmed that information processing in dorsal horn can be changed by long periods. An intense neuron stimulation which is typical of extense trauma or inflammation is followed by exacerbated nociceptive response behavior. It's believed that this kind of hyperalgesia is mediated either by nociceptor sensitization as by dorsal horn neuroplastic changes.

Younger children are more anxious, expect more pain and have more affective pain than older ones. In a study evaluating anxiety to venipuncture authors observed that anxiety was directly related to prior experiences. Although the possibilities of biological factors interfere on sensitivity during infancy, researches on pain in children suggest that children that report greatest pain intensity may not have reached enough developmental status to let them see the purpose of pain situation or to generate any dealing strategy with pain. This explanation can justify high anxiety degree influencing pain expectance (Lander & Fowler-Kerry 1991, Capone & Aloisi 2004).

It's impossible to register pain report in animals as well as their emotional discomfort; however it's possible to estimate that anxiety by repetition of a same acute and intense stimulus in a period which memory has been poorly fed (GF3) due to short time living produced more vigorous responses with lower pain thresholds, in the same way that the idea that capability to deal with uncomfortable events modifies pain reaction (Lander & Fowler-Kerry 1991, Goodenough et al. 1999, Al-Chaer et al. 2000, Hohmeister et al. 2009).

Another important point is pain tolerance, related do individual's capability to support a stimulus. Although pain perception is uniform among healthy individuals, affective fraction of pain varies largely. This suggests that previous knowledge as well as pain situation interferes with its affective response or tolerance (Ririe et al. 2004, Walker et al. 2009).

Affective quality of tonic pain experimentally applied in human beings is more comparable to chronic pain than to phasic pain, what makes phasic pain tests most indicated to acute pain studies (Lavriere & Melzack 1996, Souza et al. 2008).

In contrast, the test used in this study produced a persistent tissue injury involving different neuronal pathways as well as pharmacological ways, reproducing clinical pain with fidelity and permitting subjects to deal with pain and contributing to memory formation (Souza et al. 2008).

Results and observations on the present study lead to a rethink of other studies about sensitivity performed with animals of age and life periods unknown. There is influence of these variables and previous noxious exposition in hyperalgesia expression.

CONCLUSIONS

Younger animals showed decreasing pattern on hyperalgesia thresholds when the same noxious stimulus was repeated.

Older animals showed an increasing pattern on hyperalgesia thresholds when the same noxious stimulus was repeated.

Formalin test applied during first third life in rats may be capable to modify pain sensitivity for long periods.

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