Published in final edited form as:

Int J Dev Neurosci. 2009 May ; 27(3): 271–277. doi:10.1016/j.ijdevneu.2008.12.006.

Impact of age and strain on ischemic brain injury and seizures after carotid ligation in immature mice

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Abstract

Stroke is an important cause of neurologic injury in the neonatal period and frequently results in lifelong neurologic impairments. We reported previously that unilateral carotid ligation on postnatal day (P)12 in CD1 mice causes acute behavioral seizures and unilateral brain injury and provides a model for neonatal stroke in human infants. In the present study we confirmed that behavioral seizures observed after ligation on P12 in the CD1 strain are associated with rhythmic ictal discharges that show temporal progression on electrocorticograms. We also examined the effects of carotid ligation performed at different ages in CD1 mice or performed in the C57Bl/6 strain. The right common carotid was ligated at P7, P10, P12 or P21 in CD1 mice or at P12 in C57Bl/6 mice. Littermate controls received sham surgery. Seizures were rated for 4 h after surgery; brain injury was scored one week later. In a separate group of P12 CD1 mice, electrocorticographic activity was recorded continuously for 4 h after carotid ligation or sham surgery. Brain injury and cumulative seizure score varied significantly with age ($p < 0.001$) and strain ($p < 0.001$). In CD1 mice, injury was greatest after ligation on P10 to P12 and seizure score was maximal at P12. Seizure scores were significantly correlated with injury after ligation on P10 or P12. C57Bl/6 mice, like C3Heb/FeJ mice examined previously, were much less vulnerable to seizures and injury than CD1 mice after ligation on P12. This study demonstrates that carotid ligation in the CD1 mouse on P12 causes acute electrographic rhythmic discharges that correlate with behavioral seizures. We also found that the age at which ligation is performed and genetic strain have a strong influence on the severity of injury.

Keywords

ischemia; neonatal stroke; immature brain; seizures; electrographic discharges; ECoG

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1. Introduction

Stroke is a significant cause of neurologic morbidity in neonates (approximately 1 in 4000 term births) (Lynch et al., 2002; Lynch and Nelson, 2001), infants, and children (approximately 8 cases per 100,000 children per year) (deVeber, 2002). Neonatal stroke, pediatric arterial stroke and pediatric cerebral sinovenous thrombosis often present with acute seizures (Lynch and Nelson, 2001; deVeber et al., 2000; Azam, 1998). Approximately 75% of pediatric stroke survivors have permanent neurologic deficits including hemiparesis, epilepsy, learning disabilities, visual-field deficits, and mental retardation (Koelfen et al., 1995), and those that present with acute seizures are at increased risk for poor functional outcome (Delsing et al., 2001).

Mouse models of postnatal stroke are of particular interest for investigating mechanisms of ischemic injury, neuroprotection or neuroregeneration, and for evaluating genetic or cell-based interventions. Stroke injuries are dynamic pathophysiological processes, in which the initial ischemic insult can trigger glutamate release and a cascade of biochemical reactions that lead to seizures and energy failure. Membrane depolarization, overactivation of NMDA receptors, and stimulation of voltage-sensitive ion channels lead to excessive intracellular calcium accumulation and eventually cell death (Johnston et al., 2001).

We previously observed that after unilateral carotid artery ligation under normoxic conditions, postnatal day (P)12 CD1 mice exhibit seizure-related behavior and brain injury (Comi et al., 2004). Brain injury was moderate to severe and involved the cerebral cortex, hippocampus, striatum, and thalamus. Seizure severity was strongly correlated with the extent of brain injury. Furthermore, we have shown that vulnerability to ischemic seizures and injury is less in P12 C3HeB/FeJ mice than in P12 CD1 mice (Comi et al., 2005).

Based on prior reports of age and strain-related differences in vulnerability to hypoxic-ischemic insult in rats and mice (Sheldon et al., 1998; Towfighi et al., 1997), we expected similar differences in vulnerability in this unilateral carotid ligation model of stroke in the developing brain. We report here the impact of unilateral right common carotid ligation in P7, P10 and P21 CD1 mice as well as in P12 C57Bl/6 mice and compare these findings to our previous observations in P12 CD1 and C3HeB/FeJ mice. We also determined the electrophysiological correlates of acute seizure behavior in this model by examining electrocorticographic (ECoG) activity after unilateral carotid ligation on P12.

2. Experimental Procedures

2.1 Carotid Ligation

Unilateral right common carotid ligation or sham surgery (comparable anesthesia time with skin incision but no artery dissection or ligation) was carried out under isoflurane anesthesia (4% induction, 1.2% maintenance) in P7 CD1 (n=25 ligated and 5 shams from 3 litters), P10 CD1 (n=23 ligated and 7 shams from 3 litters), and P21 CD1 mice (n=25 ligated and 5 shams from 3 litters), and in P12 C57Bl/6 mice (n=20 ligated and 2 shams from 3 litters; day of birth = P1). In each litter, 1 or 2 animals were selected randomly at the time of surgery as shams. A 2% solution of lidocaine was applied to the surgical site at the time of suturing. Mice were immediately placed in an incubator at 35° C. Previously reported data for P12 CD1 mice (n=28 ligated and 5 shams from 3 litters) and for P12 C3HeB/FeJ mice (n=22 ligated and 3 shams from 3 litters) (Comi et al., 2004; Comi et al., 2005) are included here for direct comparison to the new data.

2.2 Seizure rating

Observers unaware of ligation status scored seizure activity using a seizure rating scale for mice described previously (Comi et al., 2004). The observers assessed the behavioral features characteristic of seizures continuously for 4 h after surgery. Every five min, each animal was assigned a score for the highest level of seizure activity observed during that interval (0 = normal behavior, 1 = immobility, 2 = rigid posture, 3 = repetitive scratching, circling or head bobbing, 4 = forelimb clonus, rearing and falling, 5 = mice that exhibited level four behavior repeatedly, and 6 = severe tonic-clonic behavior). At the end of the 4 h observation period, pups were returned to the cage with their respective dam and the 5-min interval scores were summed to obtain a cumulative seizure score. One week later, mice were anesthetized with chloral hydrate and perfused with 4% formaldehyde.

This protocol was approved by the Johns Hopkins University Animal Care and Use Committee, in compliance with local, national and international standards on animal welfare.

2.3 Histopathologic evaluation of brain injury

Neuropathologic injury was examined in coronal brain sections stained with cresyl violet. Two independent assessments of brain injury were made, and the average of the two scores was assigned as the brain injury score, as previously described (Nakajima et al., 2000), with minor modifications (Hagberg et al., 2004). Injury was scored from 0 to 4 for cortex (0: no injury, 1: one to three small groups of injured cells, 2: one to several larger groups of injured cells, 3: moderate confluent infarction, 4: extensive confluent infarction) and 0 to 6 for hippocampus, striatum, and thalamus (0–3 for no, mild, moderate or extensive infarction and 0–3 for no, mild, moderate or extensive atrophy). Regional scores were summed to obtain a total brain injury score for each subject, which ranged from 0 to 22.

Kruskal-Wallis analysis with Dunn's multiple comparison test was used to determine the effect of age or strain upon brain injury or seizure score. Data from prior studies of P12 CD1 and C3Heb/FeJ mice (Comi et al., 2004; Comi et al., 2005) were included in the analyses for comparison with the mice in the present study. Non-parametric regression was used to examine the relationship between seizure score and brain injury score. Chi-square or Fisher's exact test was used to compare the percent injured in each group. P-values less than 0.05 were considered significant.

2.4 Electrocorticographic recording

Unilateral common carotid artery ligation (n=8) or sham surgery (n=2) was performed under isoflurane anesthesia in CD1 mice at P12. Immediately after ligation, stainless steel electrodes (Plastics One, Inc., Roanoke, VA) were placed in parietal cortex 2 mm lateral to the midline on each side, secured by cyanoacrylate adhesive. A third electrode was placed in the cerebellum to serve as ground. The animals recovered from anesthesia in an incubator at 35°C for approximately 20 min. ECoG signal was then recorded continuously for 4 h, using Grass-Telefactor data acquisition software (PolyView v2.5, Astro-Med, West Warwick, RI), with analog signal conditioning (500-fold amplification and band-pass filtering at 0.3–70 Hz) prior to digital conversion and storage for offline analysis. Behavior was continuously observed and video recorded, noting the time and score for any seizure behavior as described above.

Analysis of ECoG data was performed using Reviewer, a data review module of the PolyView software. The ECoG signal was low-pass filtered using a second-order Butterworth filter with a 30 Hz cutoff frequency to eliminate environmental artifact and 60 Hz noise. The ECoG recordings were visually inspected, and episodes of rhythmic discharges were counted and analyzed to determine discharge rate. We also examined the temporal correlation between behavioral seizure scores and electrocorticographic episodes of rhythmic discharges.

3. Results

3.1 Electrocorticographic activity after unilateral carotid ligation

Electrocorticographic recordings performed in eight P12 CD1 mice after unilateral carotid ligation revealed rhythmic discharges in 7 of these animals. Six out of 8 ligated mice (No. 1, 2, 4, 5, 6 and 7) displayed paroxysmal rhythmic discharges during the first hour of ECoG recording (which began approximately 20 min after surgery), mice 1 and 6 displayed such activity during the second hour, mice 2, 5 and 6 during the third hour, and mice 6 and 8 showed similar patterns during the fourth hour. The number of recorded episodes of electrographic ictal activity per animal ranged from 3 to 12, and the episode duration ranged from 10 to 400 seconds. The ictal discharge rate ranged from 1 to 4 per second. No such activity was observed in the 2 non-ligated control mice. One of the ligated mice was withdrawn at the end of the second hour of recording due to dislodged electrodes; another died during the second hour of recording.

ECoG activity was recorded for 4 h in the remaining 6 ligated mice and 2 controls. During some portions of the recordings, when repetitive circling or tonic-clonic seizure behavior occurred, motion artifact obscured ECoG data. Immediately before and after such behaviors, however, the mice were immobile, often with the forelegs extended and the head turned to the right. Paroxysmal rhythmic electrophysiological discharges were observed during these periods of relative inactivity. In Fig. 1, a typical 20 sec ECoG recording in a sham P12 mouse (a) is compared with the temporal evolution of rhythmic discharges before and during an epoch of behavioral seizure activity in a ligated mouse (b). This animal had 7 behavioral seizure episodes within the 4 h recording period; seizure scores during these episodes ranged from 3 to 6. Most lasted from 4 to 9 min, but the final episode was prolonged, lasting 35 min. The behavioral seizure episodes were separated by quiet intervals lasting 30 to 60 min. One such episode began 3 h 19 min after the beginning of the recording, during normal quiet behavior; during this episode the recording shown in Fig. 1b was obtained. The behavioral seizure episode began with twitches every 3 to 4 seconds for 33 sec, followed by a 29 sec period of circling behavior and then a generalized tonic clonic episode that lasted nearly a minute. ECoG activity was then suppressed for 12 sec; the record in Fig. 1b begins at that point. During a period of immobile behavior we observed evolution of the ECoG rhythmic discharge activity for more than a minute before the animal entered another tonic clonic episode that lasted 50 sec (beginning at the asterisk at 3:22:26). At the end of this tonic clonic episode (3:23:15), ECoG activity was again suppressed.

3.2 Effect of age and strain on neuropathologic score

Overall, 132 of 143 ligated animals survived to perfusion for analysis of injury, and the mortality rate was not significantly different among the age and strain groups (Table 1). All sham treated animals survived (n=22). Nissl-stained sections at 3 brain levels obtained one week after ligation are shown in Fig. 2; for each ligation age or strain, a subject with a total injury score near the median is shown.

The severity of brain injury varied significantly among CD1 mice ligated at different ages ($p < 0.001$ for total brain injury score and for injury scores in each of the 4 regions examined, Kruskal-Wallis analysis). Dunn's multiple comparison test for animals ligated at different ages showed that CD1 mice ligated on P12 had significantly more total and regional brain injury than those ligated on P7 and those ligated on P10 or P12 had significantly more injury than those ligated on P21. Box plots showing the distribution and median total brain injury score after carotid ligation at each age for CD1 mice and at P12 for C57Bl/6 and C3HeB/FeJ mice are shown in Fig 3; regional injury scores are shown in Fig. 4.

Seizure scores in CD1 mice also differed significantly after ligation at different ages ($p < 0.001$, Kruskal-Wallis), with higher seizure scores after ligation at P12 than at P7 ($p < 0.05$) or P21 ($p < 0.001$, Dunn's multiple comparison test). In ligated CD1 mice (pooled across all ages), seizure score correlated with brain injury (Spearman's $\rho = 0.696$, $p < 0.001$). The correlation between brain injury and seizure score was strongest in CD1 mice ligated on P12 (Spearman's $\rho = 0.835$, $p < 0.001$); after ligation on P10 the correlation was somewhat weaker but significant (Spearman's $\rho = 0.661$, $p < 0.01$). At P7 and P21, the number of injured mice was too small for separate regression analysis in those groups.

In the CD1 mice ligated on P7, 2 animals died, one at surgery and the other during the week following ligation (seizure score=37). Most P7 mice were uninjured and had seizure scores of 0; a few had low seizure scores (range 6–15) representing brief circling or scratching; shams all had seizure scores of zero. In the 5 moderately to severely injured animals, the regional distribution of injury was similar to that seen in P12 CD1 mice (these outliers are indicated by asterisks in Fig. 4) and four of these injured animals had seizure scores greater than 20.

In CD1 mice ligated on P10, 1 animal died during the week following ligation. This animal's seizure score was high (88), but it is excluded from the analysis because it did not survive. Among the animals that survived, the distribution and severity of injury were comparable to those observed after ligation on P12. The hippocampus and cortex were most severely injured, but most animals had some injury in all 4 regions examined (Fig. 4). Seizure scores ranged from 0–74.

Three of the CD1 mice ligated on P21 died; interestingly, two of these died during the acute seizure scoring period after seizing extensively, the third also had a high seizure score and died later. Of the remaining mice only 3 were injured, and the regional distribution of injury in those cases was similar to that observed after ligation on P12 (Fig. 4). Seizure scores in the injured animals were 0, 6 and 96.

There were also striking differences among the three strains examined for brain injury and seizure severity after ligation on P12 ($p < 0.001$, Kruskal-Wallis analysis). Dunn's multiple comparison test showed that all brain injury measures (total and regional injury scores, Figs 3 and 4) and cumulative seizure scores were significantly greater in CD1 mice than either C57Bl/6 or C3HeB/FeJ mice ligated on P12. There was no significant difference between C57Bl/6 and C3HeB/FeJ in any of these measures. In C57Bl/6 mice, 6 of 20 cases were in the mild to moderate injury range (total brain injury 6 to 16.5). In these injured animals, the greatest and most frequent injury occurred in hippocampus, rather than in both hippocampus and cortex (Fig 4). Seizures scores were very low in C57Bl/6 mice (range 0–14).

4. Discussion

We have shown previously that ischemic brain injury occurs in immature CD1 mice subjected to a unilateral right common carotid artery ligation on P12 (Comi et al., 2004). In the present study, we examined the effects of unilateral carotid ligation at a range of ages in CD1 mice and at P12 in C57Bl/6 mice, a commonly used inbred strain often employed as the background strain for genetically modified mice. We found that vulnerability to injury and seizures varied markedly with age; mice were much less likely to be injured after ligation on P7 or P21 than on P12. This contrasts with the more commonly used hypoxic ischemic model that combines carotid ligation with a subsequent period of hypoxia; in that model significant injury has been reported in CD1 mice at P7 (Sheldon et al., 1998; Hagberg et al., 2004).

One interesting feature of this model is that the severity of seizures in the first four hours after injury correlates with the magnitude of brain injury seven days later. This correlation was strong and highly significant after ligation on P10 or P12 in CD1 mice. The present study also

demonstrated that acute seizure behavior after unilateral carotid ligation on P12 is associated with runs of paroxysmal rhythmic activity consistent with an ictal pattern. Studies evaluating interactions between seizures and hypoxic-ischemic brain injury in developing rats have produced mixed results. Status epilepticus induced by the GABA antagonist bicuculline after hypoxia-ischemia did not worsen brain damage in P7 rats (Cataltepe et al., 1995). In contrast, kainic acid administration in P10 rats subjected to unilateral carotid ligation and brief hypoxia triggered seizures and significantly exacerbated brain injury (Wirrell et al., 2001). In the mouse carotid ligation model, the ischemic insult is sufficient to induce both seizures and injury after ligation at P10-P12. We have reported previously that doses of gabapentin sufficient to suppress acute seizure behavior also reduce brain injury in this model (Traa et al., 2008), supporting the hypothesis that some of the mechanisms underlying acute seizures after this ischemic insult may contribute to brain injury.

Clinical experience suggests that the immature human brain is more susceptible to seizures than the mature brain, with the highest incidence of seizures in the first year of life (Hauser, 1994). Seizures that occur in the context of ischemic or hypoxic-ischemic insult are of special concern, as the severe metabolic stress of the insult combined with increased neuronal activity during seizures may result in neuronal injury. In infants subjected to hypothermic circulatory arrest or low-flow cardiopulmonary bypass for cardiac surgery, transient postoperative electrographic seizures are associated with MRI abnormalities and significantly lower psychomotor development index scores (Bellinger et al., 1999; Rappaport et al., 1998).

In the rat hypoxia model, vulnerability to epileptiform EEG is greatest at P10–12 (Jensen et al., 1991), the age of peak vulnerability to both seizures and injury in the present study. Increased seizure susceptibility is thought to result from a combination of enhanced excitation and decreased inhibition in the immature brain (Jensen, 2006). Age-related differences in receptor subunit and ion channel expression may affect vulnerability to both seizures and ischemic brain injury (Sucher et al., 1995; Sanchez et al., 2001). In addition, several studies have shown that the formation of inhibitory synapses lags behind that of excitatory synapses in cerebral cortex of rats (Blue and Parnavelas, 1983; White et al., 1997) and in CD1 mice (De Felipe et al., 1997), and the period during which excitatory circuitry predominates is comparable to the peak period of vulnerability observed in the present study.

After ligation on P12, we also found strain-related differences in seizure and injury susceptibility in C57Bl/6 mice, compared to our previous observations in CD1 and C3HeB/FeJ mice. C57Bl/6 mice, like the C3HeB/FeJ strain, were much less vulnerable to seizures and brain injury after unilateral carotid ligation than CD1 mice at P12. C57Bl/6 mice are of particular interest, given that this is a widely used background strain for genetic modification. Our findings show that in this ischemic model, C57Bl/6 and C3HeB/FeJ mice have much less vulnerability to injury and seizures than CD1 mice at P12. Differences in cerebrovascular anatomy, in particular the presence and patency of the posterior communicating arteries, have been observed in adult mice, both within and among strains (Barone et al., 1993; Kitagawa et al., 1998; Beckmann, 2000). Such differences in vascular anatomy may contribute to strain-related differences in vulnerability to bilateral carotid ligation or MCA occlusion (Barone et al., 1993; Kitagawa et al., 1998). However, strain differences in vulnerability to ischemic insult remain under conditions in which hemodynamic variables are comparable (Fujii et al., 1997; Majid et al., 2000; Wellons, III et al., 2000), indicating that other factors may contribute to differential vulnerability. In particular, strain differences have been shown in vulnerability to excitotoxic injury; adult C57Bl/6 mice are relatively resistant to injury after intrahippocampal delivery of kainic acid or quinolinic acid (Schauwecker, 2002). In contrast, C57Bl/6 mice are highly vulnerable to febrile seizures at P10–14 (van Gassen et al., 2008). Further studies of genetic, molecular, metabolic and developmental differences among these strains may help to identify factors that determine susceptibility to ischemic seizures and ischemic brain injury.

Conclusion

Unilateral carotid ligation in the developing mouse provides a model for studying aspects of ischemic injury uniquely relevant to the immature brain. The ECoG studies reported here show that electrocorticographic rhythmic discharges occur acutely after unilateral carotid ligation in P12 CD1 mice and confirm that epochs of observed seizure behavior are ictal. Ischemic seizures are a unique feature of this model that mirrors the developmental susceptibility to seizures after stroke in human neonates. We have identified striking developmental differences in vulnerability to both seizures and brain injury after ischemic insult, and further studies of differences in underlying mechanisms across this age range should be helpful in developing neuroprotective strategies.

Acknowledgements

This work was supported by National Institutes of Health Grants NS028208, K12NS001696 and 5K02NS052166-02.

Abbreviations

P	postnatal day
ECoG	electrocorticogram
NMDA	<i>N</i> -methyl-D-aspartate

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Fig. 1.

ECoG recording from a P12 CD1 mouse subjected to sham surgery (a), compared with that obtained in a unilaterally ligated mouse (b). The ECoG record from the control (a) is a 20 s sample that shows the normal range of amplitude and the uncorrelated activity typical of a P12 mouse. The tracing in (b) begins during a long epoch of seizure activity observed after carotid ligation, when ECoG activity was suppressed following a tonic clonic episode and the mouse was immobile. Rhythmic discharges began while the mouse remained immobile, and there was temporal progression in the discharges. At 3:22:26 (*) in panel b, tonic clonic behavior resumed and continued until both behavior and ECoG returned to a suppressed level in the last line of the recording. (Vertical scale in μV .)

Fig. 2.

Effect of age at the time of ligation or strain upon histopathology, one week after unilateral common carotid ligation. a) Histopathology in CD1 mice after ligation at different ages. Sections at the level of the striatum, rostral hippocampus, and caudal hippocampus show marked ischemic brain injury after ligation on P10 or P12, but little to no injury after ligation on P7 or P21. b) Histopathology in C57Bl/6 and C3HeB/FeJ mice, one week after ligation on P12. Carotid ligation in these strains produces much less brain injury than that observed after ligation on P12 in CD1 mice. (Cresyl violet Nissl-stain, subjects shown are at or near the median total brain injury for their age and strain.)

Fig. 3.

Effect of age at the time of ligation or strain on total brain injury after unilateral carotid ligation. Box plot of total brain injury, evaluated one week after ligation at different ages in CD1 mice or at P12 in other strains. The box shows the interquartile range (25th to 75th percentile), and the bold line indicates the median. Statistical outliers are indicated by an asterisk and extreme values by a circle. Total brain injury was significantly different after ligation at different ages in CD1 mice ($p < 0.001$, Kruskal-Wallis). Dunn's multiple comparison analysis showed that brain injury was significantly greater after ligation on P12 than on P7 ($p < 0.05$) or P21 ($p < 0.001$), and greater after ligation on P10 than on P21 ($p < 0.01$). Total brain injury was also significantly different among the three strains examined after ligation on P12 ($p < 0.001$, Kruskal-Wallis). Brain injury in P12 CD1 mice was significantly greater than that observed in P12 C57Bl/6 mice ($p < 0.05$) or C3HeB/FeJ mice ($p < 0.001$).

Fig. 4.

Effect of age at the time of ligation or strain on regional brain injury after unilateral carotid ligation. Box plot of regional brain injury, examined one week after ligation at different ages in CD1 mice or at P12 in other strains. Regional brain injury was significantly different after ligation at different ages in CD1 mice ($p < 0.001$ for age in each region, Kruskal-Wallis). All four brain regions examined were most vulnerable to injury after ligation on P10–12 (P12 vs. P7: $p < 0.05$ for all areas except $p < 0.01$ for cortex; P10 vs. P21: $p < 0.01$ for all areas except $p < 0.05$ for striatum; P12 vs. P21: $p < 0.001$ for all areas; Dunn's multiple comparison test). Regional brain injury was also significantly different among the 3 strains examined after ligation on P12 ($p < 0.001$, Kruskal-Wallis). In all four regions examined, the median brain injury was significantly greater in P12 CD1 mice than in P12 C57Bl/6 mice ($p < 0.01$ for all areas except $p < 0.05$ for hippocampus) and significantly greater in P12 CD1 mice than in P12 C3Heb/FeJ mice ($p < 0.001$ for cortex and thalamus and $p < 0.01$ for hippocampus and striatum, Dunn's multiple comparison test).

Table 1**Age and strain groups**

Strain	CD1	CD1	CD1 [*]	CD1	C57Bl/6	C3HeB/FeJ ^{**}
Age	P7	P10	P12	P21	P12	P12
<i>n</i> ligated	25	23	28	25	20	22
Male <i>n</i> (%)	8 (32)	14 (61)	15 (54)	11 (44)	11 (55)	11 (50)
Mortality <i>n</i> (%)	2 (8)	1 (4)	4 (14)	3 (12)	0	1 (5)
Injured <i>n</i> (%)	6 (26)	15 (68)	17 (71)	3 (14)	9 (45)	4 (19)

* Comi et al., 2004;

** Comi et al., 2005.