Investigation of antihypothalamus and antipituitary antibodies in amateur boxers: is chronic repetitive head trauma-induced pituitary dysfunction associated with autoimmunity?

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Abstract

Objective
Current data clearly demonstrated that sports related chronic repetitive head trauma due to boxing might result in hypopituitarism. However the mechanism of sports related traumatic brain injury (TBI) induced pituitary dysfunction is still unclear. In order to understand whether autoimmune mechanisms could play a role in the pituitary dysfunction due to sports related head trauma, we investigated the presence of antipituitary antibodies (APA) and antihypothalamus antibodies (AHA) in amateur boxers.

Patients and Design
Sixty-one actively competing (n=44) or retired (n=17) male boxers (mean age, 26 yr; range, 17-53) who had been evaluated regarding pituitary functions previously. In all boxers and in 60 age/sex similar normal controls AHA and APA were investigated by an indirect immunofluorescence method.

Results
AHA were detected in 13 out of 61 boxers (21.3%), and APA were detected in 14 out of 61 boxers (22.9%), but none of the normal controls. Pituitary dysfunction was significantly higher in AHA positive boxers (46.2 %) when compared with AHA negative (10.4 %) boxers (P=0.003). There was a significant association between AHA positivity and hypopituitarism due to boxing (odds ratio: 7.37, 95 % CI 1.8-30.8). There was not any significant association between APA positivity and hypopituitarism.

Conclusions
This study demonstrates for the first time the presence of AHA and APA in boxers who exposed to sports related head trauma. Moreover present investigation provides preliminary evidence that AHA are associated with the development of pituitary dysfunction in boxers, thus suggesting that autoimmunity may have a role in the pathogenesis.
Introduction

Traumatic brain injury (TBI) which is an important public health problem has been recently recognized as a leading cause of pituitary dysfunction. Hypopituitarism due to TBI may be complete or partial and 25-50% of patients has been reported to have some degrees of pituitary dysfunction even after mild TBI (1-3). The most common cause of TBI is the road traffic accidents which are responsible for more than half of the head injuries (4).

It has been reported that concussion is the main type of lesion after TBI and in 40% of these patients concussion was found (5,6). Concussion is an injury associated with sports including boxing, kickboxing, football and ice-hockey (7,8). Current data clearly demonstrated that sports related chronic repetitive head trauma due to amateur boxing and kickboxing might results in pituitary hormone deficiencies, isolated GH deficiency in particular (9-11).

Several mechanisms have been suggested for the hypothalamic-pituitary dysfunction due to TBI including hypoxic insult or direct mechanical injury to the hypothalamus, pituitary stalk, or the pituitary gland; compression from hemorrhage, edema, or increased intracranial pressure; and vascular injury to the hypothalamus or the pituitary gland (2,12). However none of these mechanisms have been proven and the mechanism of sports related head trauma-induced pituitary dysfunction is still unclear. A possible role of autoimmunity could be suggested by studies in animals which demonstrated naturally occurring IgG autoantibodies against to dying neurons in the injured brain of adult rats following a cortical lesion (13) and autoreactive antibodies against neurons and basal lamina in serum of rats submitted to experimental TBI (14). Supporting these experimental findings in a very recent clinical study significant association between antipituitary antibodies (APA) positivity and hypopituitarism 3 years after TBI has been clearly demonstrated (15).
Although the occurrence of APA in patients with TBI has been demonstrated there is no study investigating the presence of anti-hypothalamus antibodies (AHA) and/or APA in athletes exposed to chronic repetitive head trauma. In order to investigate whether autoimmune mechanisms could play a role in the pituitary dysfunction due to sports related head trauma, we have planned this study aimed at investigating the presence of APA and AHA in active or retired amateur boxers.
Subjects and Methods

After obtaining permission from the Turkish Boxing Federation, we approached all amateur, elite boxers on the Turkish National Boxing Team. We included 61 actively competing (n=44) or retired (n= 17) male boxers (mean age, 26 years [range, 17 to 53 years]). Retired boxers were official trainers and were not actively boxing. The ethics committee of Erciyes University Medical School, Kayseri, Turkey, approved this study, and we obtained informed consent from each participant. None of the boxers reported any comorbid conditions or previous pituitary disorders, and none was currently taking any medications.

Assessment of pituitary function by basal hormone levels and dynamic tests:

The data regarding pituitary functions of these subjects were recently published (11).

Gonadotropin deficiency (FSH/LH) was defined by both basal total and free testosterone levels below the normal range (total testosterone < 134 ng/dl and free testosterone < 11.5 ng/mL) in the presence of normal or low values of gonadotropins (16,17). TSH deficiency was defined by low serum fT4 level (<7.7 pg/ml) without appropriate elevation in serum TSH (16,17).

To assess GH-IGF-I axis GHRH + GHRP-6 test and glucagon stimulation tests were used, and to assess ACTH deficiency glucagon stimulation test was performed as described previously. The details of the tests and the cut-off values were recently published (10,11).

Analytic methods of Hormonal Parameters

Serum GH levels were measured by using immunoradiometric assay (IRMA) with commercial kit (DSL, Webster, Texas, USA) intra-assay and inter-assay coefficients of variation (CV) were 3.1 % and 5.9%, respectively. The minimum detection limit was 0.01 µg/L, and GH standards were calibrated according to the WHO reference standard 88/624.
IGF-I level was measured by IRMA after formic acid-ethanol extraction (DSL, Webster, Texas, USA); intra-assay and inter-assay CV were 3.4% and 8.2%, respectively.

All the other serum hormones were measured by using radioimmunoassay (RIA), IRMA or chemiluminescent methods with the commercial kits.

**Immunological evaluation**

In 61 boxers and in 60 male age–similar (mean age, 25 years [range, 18 to 50 years]) normal controls AHA and APA were investigated. In particular, AHA were detected by an indirect immunofluorescence method on cryostat section of young baboon hypothalamus supplied by Biosystem, Italia, SRL (SanMartino,Buon Albergo VR, Italy) as previously described (18).

APA were investigated by an indirect immunofluorescence method on cryostat section of young baboon pituitary gland supplied by Biosystem as previously described (19). The collaborators performing the immunological evaluation were blinded to the pituitary deficiency status of the boxers.

Control group was recruited from healthy volunteers from Turkey who had no traffic accident history, no combative sports history and no previous hospitalization history due to head trauma. Additionally if the controls declared any kind of simple head trauma including during childhood period, they were not included in the control group.

**Statistical analysis**

Statistical analysis was performed by using the SPSS 10.0 program. All data were subjected to the Kolmogrov–Smirnov test for normality. The differences between the groups were compared by unpaired t-test, and not normally distributed two groups were compared by Mann-Whitney U. The categorical data were shown as percentages and compared with χ² test. Odds ratios (OR) and 95 % confidence intervals (CI) were calculated. \( P<0.05 \) was considered
statistically significant. In addition, Pearson or Spearman’s correlation analysis to determine whether significant correlations existed between chosen variables.
Results

The behavior of AHA in all boxers and normal controls is depicted in Fig. 1A, whereas immunostaining of AHA positive and AHA negative sera are depicted in Fig. 1B. AHA were detected in 13 out of 61 boxers (21.3%), but none of the normal controls. Among the 13 AHA positive patients, 4 (30.7%) were weakly positive (titer = 1/8), while 9 (69.3%) were strongly positive (titer ranging from 1/16 to 1/64). The behavior of APA in all boxers and normal controls is depicted in Fig. 2A, whereas immunostaining of APA positive and APA negative sera is depicted in Fig. 2B. APA were detected in 14 out of 61 boxers (22.9%), but none of the normal controls. Among the 14 APA positive patients, 6 (42.8%) were weakly positive, while 8 (57.2%) were strongly positive.

Eleven of 61 (18.3 %) boxers had hypopituitarism. Three of 44 (7 %) active boxers had hypopituitarism whereas 8 of 17 (47 %) retired boxers had hypopituitarism. Baseline characteristics, deficient pituitary hormones, and individual AHA and APA titers in boxers with hypopituitarism are shown in Table 1. The boxers with pituitary dysfunction were not on hormone replacement therapy during the study period. Among the 11 boxers with hypopituitarism 6 boxers (54.5 %) had AHA positivity and 3 boxers (27.3 %) had APA positivity. All the AHA and APA titers in boxers with hypopituitarism were strongly positive (titer ranging from 1/16 to 1/64) (Table 1). Additionally when mean AHA and APA titers were compared between boxers with and without hypopituitarism; both AHA (P<0.027) and APA titers (P<0.006) were significantly higher in boxers with hypopituitarism.

The comparison of the chronic repetitive head trauma-induced pituitary dysfunction development between AHA positive and AHA negative boxers was summarized in Table 2a. Pituitary dysfunction was significantly higher in AHA positive boxers (46.2 %) when compared with AHA negative (10.4 %) boxers (P=0.003). The corresponding odds ratio was 7.37 (95 % CI 1.8-30.8), showing that the relative chance of having pituitary dysfunction after
boxing increased 7.37 fold when the subject had positive AHA. However there was not any significant difference in pituitary dysfunction development between APA positive and APA negative boxers (Table 2b).

When we performed correlation analysis of AHA and APA titers versus baseline characteristics and hormonal parameters in 61 boxers (17 retired and 44 active boxers), there was no any significant correlation (data not shown). In the subgroup analysis which included retired boxers there was a significant negative correlation (r=-0.537, P=0.026) between AHA titer ratio (higher titer ratio means low AHA titer) and retirement age, showing that high AHA titers were associated with high retirement age. Additionally there was a significant positive correlation (r=0.632, P=0.006) between AHA titer ratio and peak GH response to GHRH+GHRP-6 test, showing that high AHA titers were associated with low GH response to GHRH+GHRP-6 test in retired boxers.
Discussion

This study demonstrates for the first time the presence of AHA and APA in active or retired amateur boxers who exposed to sports related head trauma. Moreover present investigation provides preliminary evidence that AHA are associated with the development of pituitary dysfunction in boxers.

After TBI substantial amount of patients has been reported to have some degrees of pituitary dysfunction, and isolated pituitary hormone deficiencies are more frequent than multiple hormone deficiencies (1-3,20). Several possible mechanisms have been suggested for hypothalamic-pituitary dysfunction due to TBI including hypoxic insult or direct mechanical injury to the hypothalamus, pituitary stalk, or the pituitary gland; compression from hemorrhage, edema, or increased intracranial pressure; and vascular injury to the hypothalamus or the pituitary gland (2,12). In an elegant histopathological study, Salehi et al. have investigated the pituitary specimens of 42 TBI patients who died within 1 week of the motor vehicle accident. They have demonstrated acute pituitary infarcts of varying size in nearly half of the patients clearly suggesting that in the acute phase of TBI vascular damage is the most likely explanation for the early pituitary abnormalities (21). A possible role of autoimmune process involving the hypothalamic- pituitary region triggered by head trauma might be suggested by studies in animals. In an experimental study naturally occurring IgG autoantibodies against to dying neurons in the injured brain have been detected in adult rats following a cortical lesion, and it has been proposed that autoantibody binding may participate in the phagocytosis and removal of the injured neurons (13). Furthermore autoreactive antibodies against neurons and basal lamina have been found in serum following experimental TBI in rats. The authors concluded that presence of autoreactive antibodies against neurons and basal lamina after TBI could play a pathogenic role in the delayed neuron degeneration (14).
Regarding human studies presence of APA and AHA has been shown in patients with Sheehan’s syndrome even many years after the onset of hypopituitarism. The authors concluded that an autoimmune process involving both the hypothalamus and pituitary gland may contribute to late pituitary dysfunction (22). Moreover, APA can be present not only in patients with suspected autoimmune pituitary diseases, but also in those with pituitary diseases secondary to other non autoimmune specific causes, as pituitary adenoma and in some healthy subjects. However, in these cases, APA are usually present at low titers (<1:8) and they can be considered negative; on the contrary, in the sera of patients with lymphocytic hypophysitis, only when they are present at high titer (>1:8) they are considered positive, as already observed for other autoantibodies (23). Although the nature and the clinical significance of these autoantibodies are still controversial they are considered as a marker of pituitary impairment when they were detected at high titer and are undetectable (<1:8) in healthy control subjects (19,24). Apart from childhood GH deficiency APA have been associated with adulthood GH deficiency with or without related autoimmune diseases, and with autoimmune thyroiditis with normal pituitary function (19,25-27). However the association of high titers of APA (>1:8) with GH deficiency and inverse correlation between titer of antibodies and response of GH to insulin tolerance test was previously observed (19). Recently APA, specifically against GH producing cells, were found in 30% of children with idiopathic GH deficiency, prompting the authors to confirm that GH deficiency may be caused by an autoimmune process involving the pituitary gland (26). The first clinical study demonstrating the presence of APA in TBI patients 3 years after head trauma was published by our collaborative group (15). Twenty-nine patients with TBI, mainly due to road traffic accidents, were included in this study and pituitary dysfunction development ratio was significantly higher in APA positive patients (46.2 %) when compared with APA negative ones (12.5 %) (p=0.04). There was a significant association between APA positivity and
hypopituitarism due to TBI (odds ratio: 2.25, 95% CI 1.1-4.6) (15). We re-analyzed and evaluated the association between AHA positivity and hypopituitarism in these 29 TBI patients but could not find any significant association (P=0.9) (unpublished data).

The pattern of head trauma in sports related TBI, boxing and kickboxing in particular, is characterized by chronic repetitive head trauma with low intensity. In a current study from our clinic, investigation of pituitary function in amateur national boxers revealed that the retired boxers had a high rate of pituitary dysfunction (11). While the emerging evidence clearly demonstrates that athletes dealing with contact sports have a substantial risk for pituitary dysfunction, the mechanism of sports related head trauma-induced pituitary dysfunction is unclear. In the present study the first important point is the presence of AHA (21.3%) and APA (22.9 %) in amateur boxers. But the only significant association was present between AHA positivity and hypopituitarism due to boxing (P=0.003). Pituitary dysfunction development was 7.37 fold higher (95% CI 1.8-30.8) in AHA-positive boxers when compared with AHA-negative boxers. The other important point in this study was the significantly higher rate of pituitary dysfunction in retired boxers, and AHA positivity was also significantly higher (29.4 %) in retired boxers when compared to active boxers (18.1 %) (P<0.05). Although the retired boxers had not been exposed to sports related head trauma for nearly 16 years, 6 of 8 retired boxers with hypopituitarism had high titers of APA and/or AHA (Table 1). Moreover, indicating the importance of chronic exposure to the head trauma, correlation analysis in retired boxers revealed that high AHA titers were significantly associated with high retirement age and high AHA titers were associated with low GH reserve in retired boxers. Present results suggest that the activation of hypothalamic-pituitary autoimmunity, as evidenced by the presence of AHA at high titers, may have an impact on the development of pituitary dysfunction after sports related chronic repetitive head trauma. The possible mechanisms of the activation of the hypothalamic-pituitary autoimmunity after TBI
or boxing remain to be clarified. Generally in patients after moderate and severe TBI, necrotic ischemic and hypoxic changes could be present not only at pituitary but also at hypothalamic level (2,28); GH-releasing hormone (GHRH) and corticotropin-releasing hormone (CRH) neurons could be highly vulnerable to injury in these cases (28,29). With this in mind it is tempting to speculate that head trauma may trigger an ongoing cascade of vascular and histopathological alterations involving mediators of inflammatory process thus favoring the immune system activation which can contribute to late pituitary dysfunction. It is important to emphasize that there may not be a unifying hypothesis such as, early pituitary hormone deficits after TBI or sports related head trauma may be due to vascular injury and late pituitary hormone deficits may be immune mediated. In the present study 3 of the 11 boxers with hypopituitarism had neither APA nor AHA positivity. Additionally previous 1 year and 3 years prospective studies showed that in some patients pituitary function may improve over time after TBI (1,20). In fact, it has been clearly demonstrated that, during the natural course of lymphocytic hypophysitis APA could sometimes spontaneously disappear and spontaneous partial or total pituitary recovery and/or mass resolution can occur (24,30). Prospective screening of the antibody negative boxers may reveal whether they recover or not. In particular, generally in patients with TBI, as a result of the primary injury, brain edema and circulatory disturbance may occur (31-33). Inflammatory mediators (cytokines, in particular IL6, free radicals, aminoacids and nitric oxide) may lead to accelerate neuronal–cell necrosis (32,34). The infundibular hypothalamic-pituitary structure due to its peculiar anatomical and vascular characteristics may be very vulnerable to these necrotic, ischemic, hypoxic changes present after TBI (29). Therefore the release of sequestered pituitary or hypothalamic antigens from necrotic hypothalamo-pituitary system after TBI or sports related head trauma may trigger autoimmune response and could lead to late post-traumatic hypopituitarism. Interestingly while there was a significant association between APA positivity and
hypopituitarism in TBI patients (15), only significant association was present between AHA positivity and hypopituitarism in boxers. An interesting point emerging from our data is the high prevalence of GH and/or ACTH deficiency and the absence of diabetes insipidus in all AHA positive patients. This seems to indicate that these antibodies may be directed to GHRH and CRH secreting cells more than to AVP secreting cells. However, an appropriate study using a double immunofluorescence method is needed and is in progress to clarify which cells are the targets of these antibodies at hypothalamic level.

The cross-sectional design of the present study is the most important limitation. To understand whether AHA are good clinical markers for the development of pituitary dysfunction, long term prospective investigation is necessary especially in AHA positive-boxers with normal pituitary function. Additionally the characterization of the hypothalamic cells targeted by these antibodies, which is in progress, could strengthen the present findings. Moreover due to the cross-sectional design of the study we could conclude that presence of AHA may contribute to the development of hypopituitarism in boxers but at present it is difficult to claim that this relationship is causal.

In conclusion, the presence of AHA and APA was demonstrated in active or retired amateur boxers who exposed to sports related head trauma. In contrast to previous findings in TBI patients only positivity for AHA was associated with hypopituitarism in boxers implying that pattern and type of the head trauma may have an impact on the level of the hypothalamo-pituitary injury. Therefore this preliminary study, showing the presence of these antibodies at high titers in some boxers with impairment of pituitary function, suggest that autoimmunity may contribute to the development of sports related head trauma induced hypopituitarism. However, longitudinal studies with high number of boxers are needed to investigate possible disappearance overtime of APA in those with pituitary dysfunction and to clarify the predictive value of AHA as a marker of pituitary dysfunction and for the possible clinical
implications. The presence of the possible association between the autoimmunity and boxing-induced hypopituitarism may provide a new point of view in this field and promote further clinical and experimental studies.

Declaration of interest: There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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1A: Anti-hypothalamus antibodies (AHA) in boxers and in 60 normal controls

- ●: Boxers with hypopituitarism
- ○: Boxers without hypopituitarism

1B: Serum sample positive for antibodies to hypothalamic cells detected by immunofluorescence in a boxer (a) in comparison with a negative control serum (b).
2A: Antipituitary antibodies (APA) in boxers and in 60 normal controls
●: Boxers with hypopituitarism
○: Boxers without hypopituitarism

2B: Serum sample positive for antibodies to pituitary cells detected by immunofluorescence in a boxer (a) in comparison with a negative control serum (b).
Table 1: Baseline characteristics of boxers with hypopituitarism and individual AHA and APA titers.

<table>
<thead>
<tr>
<th>N</th>
<th>Age (yr)</th>
<th>Boxing status</th>
<th>Boxing duration (yr)</th>
<th>Retirement age (yr)²</th>
<th>Number of bouts³</th>
<th>Deficient pituitary hormones</th>
<th>AHA titer</th>
<th>APA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>Active boxer</td>
<td>7</td>
<td>-</td>
<td>1080</td>
<td>ACTH</td>
<td>1/16</td>
<td>Absent</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>Active boxer</td>
<td>3</td>
<td>-</td>
<td>330</td>
<td>ACTH, GH</td>
<td>1/64</td>
<td>Absent</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>Active boxer</td>
<td>10</td>
<td>-</td>
<td>1000</td>
<td>ACTH</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>Retired boxer¹</td>
<td>10</td>
<td>28</td>
<td>1000</td>
<td>ACTH, GH</td>
<td>Absent</td>
<td>1/64</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>Retired boxer</td>
<td>14</td>
<td>28</td>
<td>2240</td>
<td>ACTH, GH</td>
<td>1/32</td>
<td>1/64</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>Retired boxer</td>
<td>12</td>
<td>30</td>
<td>2400</td>
<td>GH</td>
<td>1/64</td>
<td>Absent</td>
</tr>
<tr>
<td>7</td>
<td>49</td>
<td>Retired boxer</td>
<td>12</td>
<td>27</td>
<td>960</td>
<td>GH</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>Retired boxer</td>
<td>11</td>
<td>26</td>
<td>1100</td>
<td>GH</td>
<td>1/32</td>
<td>Absent</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>Retired boxer</td>
<td>9</td>
<td>25</td>
<td>1800</td>
<td>GH</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>10</td>
<td>37</td>
<td>Retired boxer</td>
<td>8</td>
<td>25</td>
<td>800</td>
<td>GH</td>
<td>Absent</td>
<td>1/64</td>
</tr>
<tr>
<td>11</td>
<td>42</td>
<td>Retired boxer</td>
<td>11</td>
<td>28</td>
<td>880</td>
<td>GH</td>
<td>1/32</td>
<td>Absent</td>
</tr>
</tbody>
</table>

¹ Retired boxers were official trainers and were not actively boxing.

² Mean years since retirement in 17 retired boxers was 16 years (range, 8–28 years).

³ Total number of championships and training fights throughout their career.
Table 2 a,b: Comparison of pituitary dysfunction development between AHA positive and AHA negative boxers (a), and between APA positive and APA negative boxers (b).

a)  

<table>
<thead>
<tr>
<th>Pituitary Dysfunction (PD)</th>
<th>AHA p (n=13)</th>
<th>AHA negative (n=48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With PD</td>
<td>6 (46.2 %)*</td>
<td>5 (10.4 %)</td>
</tr>
<tr>
<td>Without PD</td>
<td>7 (53.8 %)</td>
<td>43 (89.6 %)</td>
</tr>
</tbody>
</table>

* $\chi^2 = 8.84$ and $P=0.003$

Data are given as number and as a percentage of each category

b)  

<table>
<thead>
<tr>
<th>Pituitary Dysfunction (PD)</th>
<th>APA positive (n=14)</th>
<th>APA negative (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With PD</td>
<td>3 (21.4 %)</td>
<td>8 (17.0 %)</td>
</tr>
<tr>
<td>Without PD</td>
<td>11 (78.6 %)</td>
<td>39 (83.0 %)</td>
</tr>
</tbody>
</table>

$\chi^2 = 0.14$ and $P=0.707$

Data are given as number and as a percentage of each category