retrieve antigenicity based on the modified method of Shi et al [19]. Endogenous peroxidase activity was blocked by treatment with 3 % hydrogen peroxide. After treatment with 10 % goat serum, the sections were incubated with each primary antibody for 48 hr at 4°C. The sections were then incubated with the biotinylated anti-mouse immunoglobulin (Vector Lab.) followed by incubation with the avidin-biotin-peroxidase complex (Vector Lab.). Antibody labeling was visualized by incubation with diaminobenzidine with nickel ammonium sulfate. Normal adult human tonsil, normal adult and postnatal 10-days-old rat were used as positive controls.

Evaluation of immunostaining. For semiquantitative evaluation of immunohistochemical staining, staining intensity was scored on an arbitrary four-point scale: 0, no immuno-positive staining; 1+, weak immuno-positive staining intensity; 2+, moderate immuno-positive staining intensity; 3+, strong immuno-positive staining intensity. We counted the cells which were stained in the 2+ and 3+ degree as immuno-positive cells. The number of melanized nigral neurons was counted in one of the midbrain sections at the level of the oculomotor nucleus on each patient and control subject. The substantia nigra was arbitrary divided into three approximately equal parts, i.e., the medial, central and the lateral part[20] and the percentage of neurons

immuno-positive for Bcl-xL, Bcl-2 or Bax among the melanized neurons was calculated at each compartment. Statistical analysis was performed using the Mann-Whitney U-test.

Double-labeling; Bax with Bcl-xL or Bcl-2. Co-localization of Bax and Bcl-xL or Bcl-2 was analyzed by a confocal laser-scanning microscope (BIO-RAD microscopy division; MRC-1024) using antibodies labeled by rhodamine-avidin complex or fluorescine-streptoavidine complex.

Results. *Bcl-xL, Bcl-2 and Bax immunohistochemistry in CTL subjects.* Bcl-xL and Bcl-2 were expressed in approximately half of the melanized nigral neurons (Fig. 1a, 1c) whereas Bax was expressed in most of the nigral neurons (Fig. 1e). Non-melanized nigral neurons also expressed Bcl-xL, Bcl-2, and Bax. These immunostaining was observed mainly in the cytosol, but Bcl-xL and Bax were also expressed in the nuclear membrane of some neurons. Bcl-xL, Bcl-2, and Bax were also expressed in most of the neurons in the oculomotor nucleus, dorsal tegmental nucleus, and the peripeduncular nucleus, but not in the neurons in the red nucleus and the superior colliculus. Some glia cells, endothelial cells, and vessel walls were positive for Bcl-xL, Bcl-2, and Bax. Pre-adsorption of antibodies studied abolished the immunostaining.

Bcl-xL, Bcl-2, and Bax immunohistochemistry in PD patients. Bcl-2 was expressed in approximately half of the melanized

nigral neurons (Fig. 1d) and Bax was expressed in most of the nigral neurons (Fig. 1f) as in the CTL subjects.

On the other hand, Bcl-xL was expressed in only one-fourth of the remaining nigral neurons as will be shown in the semiquantitative analysis later (Fig. 1b). The intensity of immunostaining of Bcl-xL-positive neurons itself was essentially similar to that of the control subjects. Both atrophic and apparently normal neurons showed similar immunostaining intensity. Lewy bodies were negative for Bcl-xL, Bcl-2, and Bax. No relationship was noted between the presence of Lewy bodies and expression of Bcl-xL, Bcl-2, or Bax in individual neurons. The glial expression of Bcl-xL and Bax was apparently increased in the SN of PD patients compared with the CTL subjects, but not in other midbrain areas. Expressions of Bcl-xL, Bcl-2, and Bax in other midbrain areas were essentially similar to those of the CTL subjects.

Semiquantitative study on Bcl-xL , Bcl-2 and Bax. In the CTL subjects, the percentages of immuno-positive nigral melanized neurons for Bcl-xL were 51.6 ± 9.9% in the medial part, 44.3 ± 7.1% in the central part, and 40.4 ± 7.1% in the lateral part, whereas in the PD patients, the percentages were 27.8 ± 7.4%, 22.7 ± 6.1%, and 20.0 ± 6.6%, respectively. The number of immuno-positive neurons was significantly smaller in the medial and the lateral parts of the SN in PD (Fig. 2b; Mann-Whitney U-test; $p<0.05$ and $p<0.02$, respectively). When the three parts of the SN were combined, Bcl-xL-positive neurons were 46.0 ± 5.9% in the CTL and 22.5 ± 5.5% in the PD patients (Fig. 2b; Mann-Whitney U-test; $p<0.02$).

The percentages of Bcl-2-positive neurons in the CTL were 62.6 ± 14.2% in the medial part, 59.4 ± 15.6% in the central part, and 70.4 ± 12.2 % in the lateral part, where as in the PD, they were 64.3 ± 9.5%, 47.0 ± 13.0% and 58.7 ± 10.3, respectively; no essential difference was noted between the two groups (Fig. 2c). When the three parts were combined, Bcl-2 positive neurons were 61.9 ± 14.7% in the CTL and 59.7 ± 10.3% in the PD (Fig. 2a). The percentages of Bax-positive neurons in the CTL were 91.0 ± 9.9% in the medial part, 88.3 ± 3.9% in the central part, and 92.3 ± 1.5% in the lateral part , where as in the PD, they were 95.6 ± 3.1%, 89.7 ± 5.0%, and 92.2 ± 5.9%; no significant difference was noted between the two groups (Fig. 2d). When the three parts were combined, Bax-positive neurons were 90.5 ± 2.6% in the CTL and 92.5 ± 4.2% in the PD (Fig. 2a).

Double-labeling; Bax with Bcl-xL or Bcl-2. Figure 3 illustrates confocal lazer images of immuno-fluorescence for Bcl-xL(Fig. 3a) and Bax (Fig. 3b), and Bcl-2 (Fig. 3c) and Bax (Fig. 3d) in the SN of PD patients. Five patterns of immunostaining were detected in nigral neurons, i.e., neurons expressing only Bcl-xL, Bcl-2, or Bax, neurons expressing Bcl-xL and Bax, and Bcl-2 and Bax. Co-localization of Bcl-xL and Bcl-2 was not tested. No significant difference was note in the overall co-localization pattern between the CTL and the PD.

Discussion. We showed that immunostaining for Bcl-xL, but not Bcl-2 or Bax, is down-regulated in the SN neurons of PD patients compared to CTL subjects. According to the literature, Bcl-xL and Bcl-2 are expressed in a

large population of neurons during embryonic development and that Bcl-2 expression is down-regulated during maturation while that of Bcl-xL is retained in the adult CNS[21-23]. Therefore, Bcl-xL may play more important role than Bcl-2 in the survival of neurons in the adult CNS[24]. Although limitations are present in the analysis of post-mortem human specimens, down-regulation of Bcl-xL expression in PD in our study suggests increased vulnerability of nigral neurons for neuronal death by apoptosis. As Bcl-2 and Bax expression in PD did not change significantly from the CTL, the down regulation of Bcl-xL does not appear to be secondary to neuronal degeneration or loss of mitochondria; Bcl-xL, Bcl-2 and Bax are expressed on mitochondrial membrane. The down regulation of Bcl-xL may be either due to decreased synthesis or increased degradation.

Apoptosis appears to play a critical role in neuronal death in many neurodegenerative disorders including Alzheimer's disease (AD) [25], Huntington disease[26], PD[3-4], herediary spinocerebellar degenerations [27], and ALS[28]. Our group[4], Anglade et al.[2], and Tatton et al.[3] reported evidence to indicate apoptosis in nigral neurons in PD TUNEL or electronmicroscopy. However, recently, Kösel et al.[7] and Banati et al.[29] reported serious doubt on the existence of apoptosis in nigral neurons in PD. Such controversies may in part be due to the lack of appropriate methods to explicitly demonstrate apoptosis in a small autopsy material like SN.

Another approach to address the question of apoptosis is the analysis of Bcl-2 family proteins. Mogi et al. reported higher level of Bcl-2 in the striatum[15] and SN (Mogi personal communication) in PD by enzyme-linked immunosorbant assay (ELISA). But in our study, neuronal expression of Bcl-2 was not increased in PD. Vyas et al. also reported

normal bcl-2 mRNA expression in nigral neurons of PD patients[30]. This discrepancy can be explained by increase in the glial expression of Bcl-2 as a whole.

Studies on Bcl-xL in human brains are limited; Shoma et al.[18] and Krajewski et al.[31] reported Bcl-xL expression in some Purkinje cells. Drache et al.[24] reported lower levels of Bcl-xL expression in AD brains by Western blotting but not by immunohistochemistry. We found significant down regulation of Bcl-xL expression in neurons in the lateral and the central parts; the areas which are more vulnerable in PD[20].

The relationship between up- or down-regulation of anti- and pro-apoptotic proteins and the degree of neurodegeneration is not simple. In Alzheimer brains, neurons exhibiting DNA fragmentation were associated with the up-regulation of Bcl-2 expression, whereas neurons exhibiting neurofibrillary tangles showed down-regulation of Bcl-2 expression[32]. On the other hand, Su et al.[33] and MacGibbon et al.[34] showed positive correlation between Bax up-regulation and tangle formation in AD. In progressive supranuclear palsy, Tortosa et al.[16] reported normal Bcl-2 and Bax immunoreactivities in the SN neurons.

It has been postulated that the ratio of Bax to Bcl-xL and Bcl-2 plays a critical role in the regulation of the relative vulnerability to apoptotic cell death[35]. On the other hand, according to Cheng et al.[36] and Knudson et al.[37] Bax, Bcl-xL and Bcl-2 are able to regulate apoptosis independently. Our double-labeling study suggests that the interaction with may not be necessary for Bcl-xL to exert its anti-apoptotic effect, and that down regulation of Bcl-xL itself could promote apoptosis in the PD patients.

In conclusion, we showed down regulation of Bcl-xL in the SN of PD patients. This did not appear to be secondary to neuronal degeneration, as expressions of Bcl-2 and Bax were essentially normal. Further studies on the synthesis and degradation of Bcl-xL appear to contribute to the elucidation of nigral neuronal death in PD. Furthermore our results warrant studies on the genetic polymorphism of the Bcl-xL gene in comparison with Bcl-2 and Bax gene polymorphisms to explore genetic risk factors for PD. (2193 words)

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divided into three approximately equal parts, i.e., the medial, the central and the lateral, and the percentage of neurons immuno-positive for Bcl-xL (b), Bcl-2 (c), or Bax (d) is calculated at each compartment. (b) The parcentage of Bcl-xL-immuno-positive neurons is significantly lowerr in the medial and the lateral parts of the SN in PD (*$p<0.05$ and **$p<0.02$ vs CTL, Mann-Whitney U-test).

Figure3. Double-labeling; Bax with Bcl-xL or Bcl-2. Co-localization of immuno-fluorescence for (a) Bcl-xL and (b) Bax , and (c) Bcl-2 and (d) Bax in the SN of PD patients (X150). The primary antibody for Bcl-xL in the figure (a) and for Bcl-2 in the figure (c) is recognized by green precipitates, whereas, Bax immunoreactivity is identified by red precipitates (b and d). The green arrow points to a cell immunoreactive for Bcl-xL or Bcl-2 alone, the red arrow points to a cell immunoreactive for Bax alone and the white arrow points to double-stained cells. The combinations of immunoreactivity can be classified into neurons stained for either Bcl-xL, Bcl-2, or Bax alone, or neurons stained for both, which form the majority.

Figure legend

Figure 1.Immunohistochemical detection of Bcl-xL, Bcl-2 or Bax (X100). Scar bar, 50 μm. (a) Bcl-xL- , (c) Bcl-2 - or (e) Bax- expression in the SN in the CTL subject; the tiny grayish spots in the cytoplasm and nuclear envelope indicate positive staining. Regarding the subcellular distribution, Bcl-xL staining is observed mainly in the cytosol, but Bcl-xL and Bax were also expressed in the nuclear membrane of some neurons. The brown spots are melanin granules in the dopaminergic neurons. (b) Bcl-xL- , (d) Bcl-2– or (f) Bax-expression in the SN in a PD patient; Bcl-xL-expression is apparently reduced, however, Bcl-2- and Bax-expression are essentially similar to those in the CTL subjects.

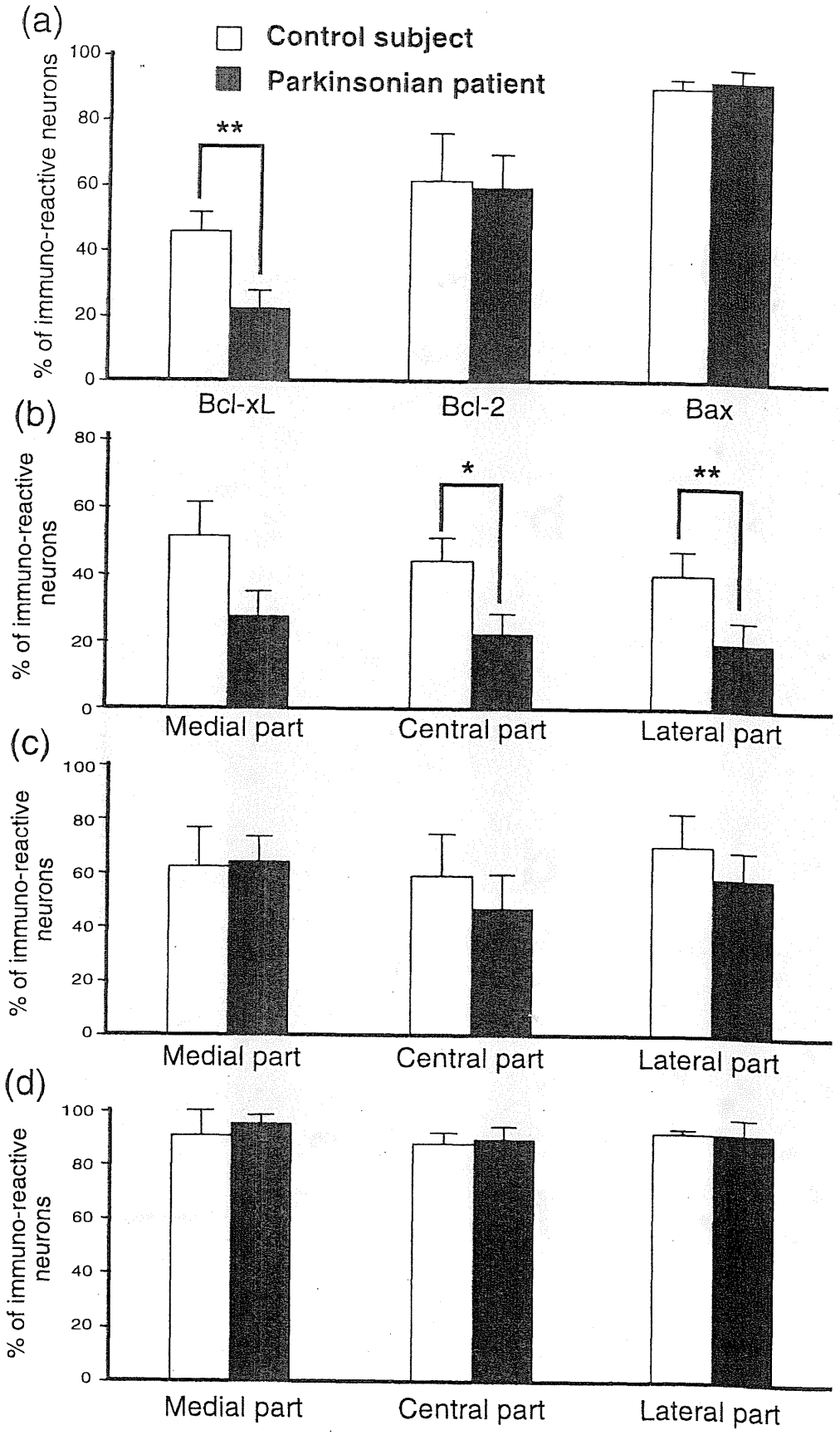
Figure2. (a) The percentages of immuno-reactive nigral melanized neurons for Bcl-xL, Bcl-2 or Bax in the CTL subjects and in the PD patients. The ordinate indicates "%" of immuno-reactive neurons counted in one of the midbrain sections at the level of the oculomotor (mean ± SEM, for n=7 for CTL and n=6 for PD). See the text for the details . The percentage of Bcl-xL-positive neurons among the melanized nigral neurons is significantly lower in the PD patients than in the CTL subjects (**P<0.02 vs CTL, Mann-Whitney U-test). (b)(c)(d) The substantia nigra is arbitrary

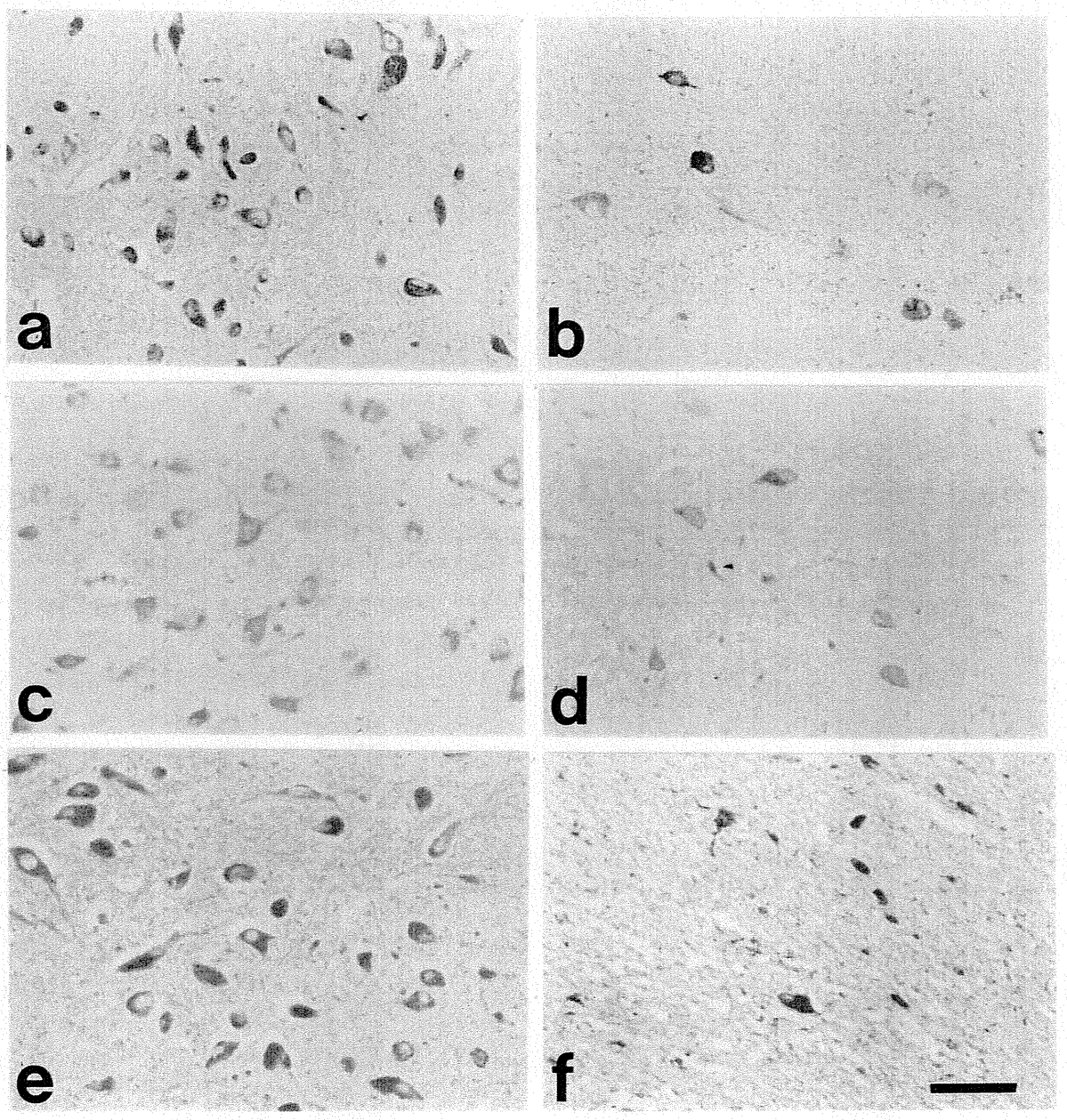
Table1 *Clinical characterisitics*

Patient number	Diagnosis	Age (yr)	Sex	Duration of the disease (yr)	Cause of Death	Postmortem interval (hr)
1	CTL	41	M		pneumonia	4.5
2	CTL	52	M		pneumonia	1.0
3	CTL	53	F		myocardial infarction	2.5
4	CTL	67	M		leukemia	3.5
5	CTL	84	M		heart failure	3.1
6	CTL	93	F		heart failure	4.0
7	CTL	76	M		heart failure	2.0
8	PD	63	F	7	pneumonia	4.5
9	PD	66	F	10	sudden death	8.5
10	PD	75	M	11	pneumonia	1.6
11	PD	76	F	18	pneumonia	3.8
12	PD	78	M	15	heart failure	8.5
13	PD	81	F	7	pneumonia	2.5

CTL=Control subjects, PD=Parkinson's Ddisease

Fig.2


(a)
Control subject
Parkinsonian patient
% of immuno-reactive neurons
100
80
60
40
20
0
**
Bcl-xL
Bcl-2
Bax
(b)
% of immuno-reactive neurons
80
60
40
20
0
*
**
Medial part
Central part
Lateral part
(c)
% of immuno-reactive neurons
100
80
60
40
20
0
Medial part
Central part
Lateral part
(d)
% of immuno-reactive neurons
100
80
60
40
20
0
Medial part
Central part
Lateral part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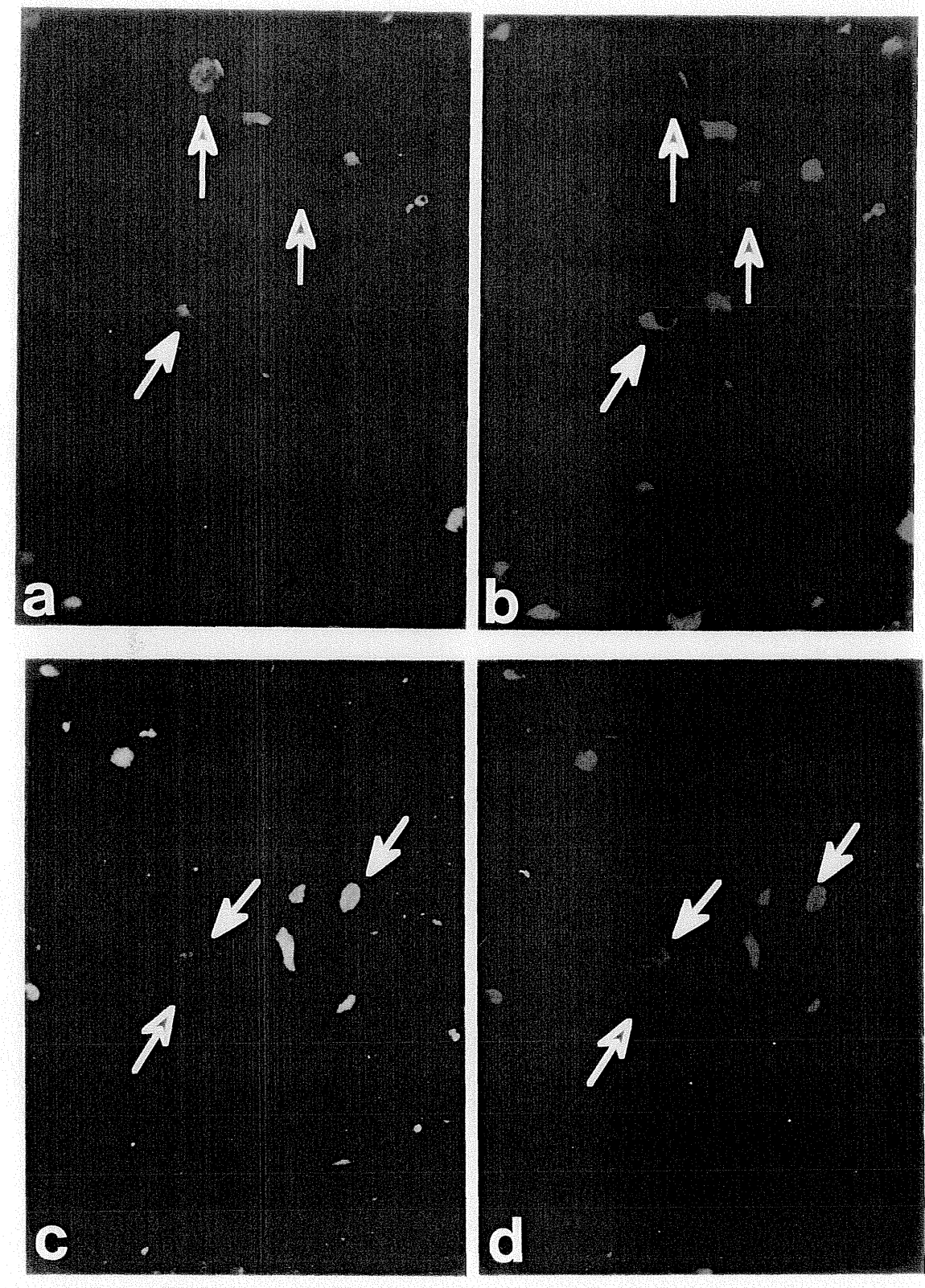
a
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Increase of 8-oxo-dGTPase (*hMTH1*) in Mitochondria of Nigrostriatum of Parkinsonian Brain

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ABSTRACT

There is growing evidence that oxidative stress and mitochondrial respiratory failure with an attendant decrease of energy output are involved in nigral neuronal cell death in Parkinson's disease (PD). 8-oxo-dGTPase (8-oxo-7,8-dihydrodeoxyguanosine triphosphatase: *hMTH1*) played an important role in the control of spontaneous mutagenesis, the production of including 8-oxo-dG (8-oxo-2,8-dihydroguanine). 8-oxo-dGTPase was increased in the mitochondria of the substantia nigra in PD patients. In the study of 8-oxo-dG, while nuclear DNA showed no modification in all subjects, mitochondrial DNA showed increased modification in PD patients. These results support the concept in oxidative mitochondrial stress in PD. Moreover, an increase of *hMTH1* appears to be a useful molecular marker of oxidative stress that can be employed to explore the relationship between such stress and genomic instability.

Key words: 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase, *hMTH1*, 8-dihydrodeoxyguanosine, oxidative stress, Parkinson's disease