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A comprehensive approach to determining BER capacities and their change with aging in *Drosophila melanogaster* mitochondria by oligonucleotide microarray

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ABSTRACT

DNA repair mechanisms are key components for the maintenance of the essential mitochondrial genome. Among them, base excision repair (BER) processes, dedicated in part to oxidative DNA damage, are individually well known in mitochondria. However, no large view of these systems in differential physiological conditions is available yet. Combining the use of pure mitochondrial fractions and a multiplexed oligonucleotide cleavage assay on a microarray, we demonstrated that a large range of glycosylase activities were present in Drosophila mitochondria. Most of them were quantitatively different from their nuclear counterpart. Moreover, these activities were modified during aging.

1. Introduction

The mitochondrial matrix is a rather harmful environment where strong oxidants are generated. These entities, termed reactive oxygen species (ROS), are produced continuously, mainly at the level of complexes I and III in the respiratory chain, during normal function, and are exacerbated during physical exercise or pathological conditions [1,2]. They are extremely detrimental to various cell structures such as proteins, lipids, and nuclear or mitochondrial DNA (mtDNA). mtDNA is directly bound to the inner membrane, and is less well protected than nuclear DNA [3]. ROS induce DNA base lesions through oxidation, deamination mechanisms and DNA strand breaks. Even though mtDNA molecules are numerous in every mitochondrion (average 2–10 copies), DNA lesions can accumulate and lead to heteroplasmy, a status where more than one type of mitochondrial genome is present in an organism. The vertebrate mtDNA is usually an extremely compact genome made up of 37 genes for essential respiratory complexes subunits, tRNA, and rRNA synthesis. Hence any DNA lesion can lead, with a high probability, to dramatic changes in mitochondrial protein expression and/or functions. Several pathologies have indeed been associated with mtDNA point mutations or deletions [4,5].

Mitochondrial DNA repair mechanisms have long been ignored or considered of low efficiency [6]. Today, mtDNA repair activities are fully acknowledged. They are efficient on single or double strand breaks in mitochondria [7–9], but mechanisms and actors remain mainly unknown. By contrast, base excision repair (BER) which is the main system handling the DNA damage resulting from oxidative insults activities, is well known and characterized in mitochondria [10,11]. Enzymatic proteins involved in this process are all nucleus-encoded. However, they are mostly identical to ac-

Abbreviations: AP site, abasic site; APE1, apurinic/apyrimidic endonuclease 1; BER, base excision repair; Cy3, Cyanine 3; Hx, hypoxanthine; NEIL1, Nei-like 1; 8oxoG, 8-oxo-7,8-dihydroguanine; NTH1, endonuclease three homolog 1; ODN, oligonucleotide; OGG1, 8-oxoguanine DNA glycosylase; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species; Tg, thymine glycol; THF, tetrahydrofuran; U, uracil; UNG, uracil DNA glycosylase

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tive molecules found in the nucleus, or are isoforms from either alternative splicing or multiple transcription starting sites [12,13].

The different steps of BER are well described in [10] and [11]. The initial steps are base damage recognition and removal, through DNA glycosylases followed by abasic site recognition and hydrolysis by an endonuclease. But the entire specificity of the process is provided by the action of the different glycosylases.

Various mtDNA damages have been observed during pathological or physiological conditions. Among all of them, aging has been well studied leading to the hypothesis that mtDNA alteration could be a result of several processes: increase of damages causes, changes in replication activities or changes in mtDNA repair capacities [14]. Indeed, it has been shown that mtDNA damage increase and mitochondrial biochemical capacities decrease, during aging [14] putting the light on a potential involvement of BER activities in these phenomena.

Most studies on mtDNA repair mechanisms during aging have focused on either the whole process without discriminating specific glycosylases [15] or on single glycosylase activities [16,17]. Such analyses were performed by oligonucleotide (ODN) cleavage assays. In this process, ^{32}P-labeled oligonucleotides displaying various base alterations were used.

A more comprehensive assay allowing investigation of BER has been developed, ensuring simultaneous measurement of several excision activities [18–22]. This new approach was made possible by the use of microarrays functionalized by fluorescent lesion-bearing oligonucleotides. Only nuclear extracts have been tested so far using this multiplexed assay.

In this study, we show that these microarrays are very convenient for the quantification of BER activities in fruitfly mitochondria. This approach was used to analyze changes in mitochondria BER capacities during aging.

2. Materials and methods

2.1. Fruitflies

Fruitflies of *Drosophila melanogaster* strain w[1118] from the Bloomington Drosophila stock center, were reared in tubes (75 fruitflies per tube) at 19–20 °C on standard axenic medium [24]. Two populations of male fruitflies were used in this study: young (4–5 days after pupal hatching) and old (8 weeks after pupal hatching).

2.2. Subcellular fractionation

Reagents were from Sigma Aldrich unless otherwise stated. The entire procedure was performed at 4 °C in isolation buffer (IB): 10 mM HEPES, pH 7.6, 0.22 M sucrose, 0.12 M mannitol, 2 mM EGTA, 5 mM dithiothreitol, protease inhibitors (cOmplete Mini EDTA-free, Roche Diagnostic). Mitochondria and purified mitoplats were prepared according to protocols previously described in [24–26] and pure nuclear fractions were obtained as in [27]. The entire process was carried out as described in Fig. 1.

Protein concentrations were determined using the Bradford method [28] (Biorad).

2.3. Western blot analyses

Mitochondrial or nuclear fractions (15 μg proteins) were run on 10% or 15% (w/v) SDS–PAGE, blotted on nitrocellulose membrane (GeHealthcare) subsequently probed with primary antibodies raised against lamin (DSHB), histone H3, Rieske protein (gift from Dr. C. Godinot, Lyon) and pyruvate dehydrogenase (PDH, Mitosciences). Antibodies dilutions were respectively 1:750, 1:2000, 1/13 500 and 1:2000. Incubations and detections were carried out as previously described [23].

2.4. Enzyme assays

Cytochrome *c* oxidase and citrate synthase assays were performed on mitochondrial or nuclear fractions according to a previously described protocol [29]. All the enzymatic measurements were carried out using a heat-controlled spectrophotometer (Shimadzu) at 28 °C.

2.5. DNA repair assays

The excision reactions were conducted using the modified oligonucleotide arrays already described elsewhere [20,21]. The principle of these oligonucleotide arrays and oligonucleotides characteristics were detailed in a previous study [20]. Briefly, each well of a 24-well slide was functionalized by a series of lesion-containing oligonucleotide duplexes (lesion ODNs) and a control duplex (control ODN, with no lesion). The duplexes were tethered in duplicate to the slide surface by one end, and labeled with a Cyanine 3 (Cy3) at the other end.

Eight lesion substrates were present: ethenoadenine (EthA) paired with T, hypoxanthine (Hx) paired with T, 8oxoguanine (8oxoG) paired with C, A paired with 8oxoG, thymine glycol (Tg) paired with A, tetrahydrofuran (THF), as abasic site (AP site) analog, paired with A, uracil (U) paired with G and U paired with A.

Excision reactions were conducted with 10 μg of protein per well at room temperature (23 °C) for 1 h in 80 μL of excision buffer (10 mM HEPES/KOH pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM $ZnCl_2$, 1 mM DTT, 0.5 mg/ml BSA). The slides were subsequently rinsed for 3 × 5 min with 80 μL of washing buffer (PBS containing 0.2 M NaCl and 0.1% (v/v) Tween 20). On each slide, two wells were incubated with the excision buffer only (control wells) to serve as a reference, arbitrarily set at a fluorescence level of 100% for the calculation of the excision rates. The spot fluorescence was quantified using a GenePix 4200A scanner (Axon Instrument, Molecular Devices, Sunnyvale, CA, USA). Total spot fluorescence intensity was determined using the GenePix Pro5.1 software (Axon Instrument). Each extract was tested twice. The results between replicates (four spots) were normalized using the NormalizeIt software as described in [20]. A first operation calculated the excision rate of each of the lesion ODNs in the extract-containing wells compared with the lesion ODNs of the control wells. A correction was then applied that took into account the possible degradation of the control ODN (no lesion). For each slide, normalized fluorescence level of control wells (repair buffer alone) was taken as reference and fluorescence level of each lesion-ODN of control well was set up to 100. Final results for each lesion ODN, expressed as percentage of cleavage, were calculated using the formula: 100 × (1−percentage of fluorescence of lesion ODN/percentage of fluorescence of control ODN).

2.6. Statistical analyses

When histograms are presented, data are expressed as mean ± standard deviation (S.D.). Five to eight different extractions were performed and tested by enzymatic assays (each in duplicate) and on oligonucleotides microarrays (each in duplicate). Statistical significance was assessed using the Student's *t* test. For all statistical tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Purified mitochondrial and nuclear fractions

As a first step prior to any repair measurement in mitochondria, we sought to obtain purified functional mitochondrial fractions with no contaminants from cytosol or nuclear compartments, in

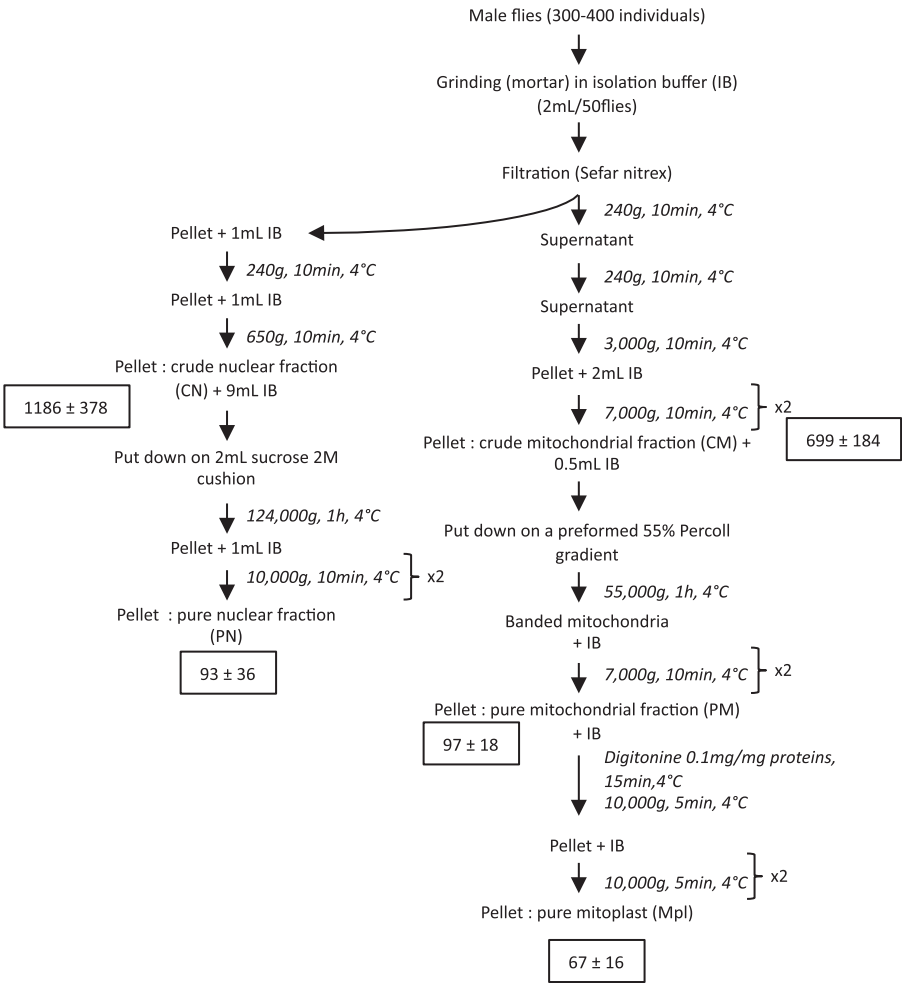

Fig. 1. Protocol details for obtaining different sub-cellular fractions. Data in boxes indicate amounts of proteins obtained from various sub-cellular fractions (in μg proteins/100 flies). Values are expressed as means ± S.D. and correspond to 5 different experiments. S.D.: Standard Deviation.

order to ensure that tested repair activities came solely from the expected compartment.

The protocol used and optimized for this process is illustrated in Fig. 1. Mitochondrial and nuclear purity was estimated using Western blot analyses with specific antibodies: anti-PDH (mitochondrial matrix), anti-Rieske (mitochondrial inner membrane, component of the respiratory complex III), anti-lamin (Dm0, nuclear envelope), and anti-histone H3 (nucleoplasm).

The crude mitochondrial fraction obtained by differential centrifugation showed, by Western blot analysis, a persistency of nuclear proteins such as lamin and histone H3, suggesting a partial nuclear contamination of this fraction, independent of fruitfly age (Fig. 2A, lane CM). A subsequent purification step from crude mitochondrial fractions on Percoll gradient (Fig. 2A, lanes PM) not only preserved the fraction integrity as shown by the presence of mitochondrial matrix protein PDH, but was also sufficient to eliminate residual traces of nuclear components. An additional treatment of these fractions with digitonin removed all residual contaminants (Fig. 2A, lanes Mpl).

Crude nuclear fractions were prepared and analyzed by Western blot (Fig. 2B). As observed for mitochondria, these fractions were still contaminated with other compartment proteins (Fig. 2B, lanes CN) such as PDH and Rieske. Another purification step using a sucrose cushion as described elsewhere [27] led to the almost complete disappearance of mitochondrial contaminants in purified nuclear fraction (Fig. 2B, lanes PN). These results prove that BER activities measured in the purified nuclear fractions were exclusively of nuclear origin.

After determining the fractions purity, mitochondrial functionality was assessed using enzyme assays (Fig. 3). Two enzyme activities were chosen: cytochrome oxidase (complex IV of the respiratory chain, Fig. 3A), and citrate synthase (a mitochondrial matrix protein from the Krebs cycle, Fig. 3B).

Change (2.6-fold) for citrate synthase activity between crude mitochondria and mitoplasts was significant ($P < 0.05$). By contrast, the increase observed for cytochrome oxidase between these two fractions (1.34-fold) with young fruitflies was not significant. It is possible that digitonin treatment could partially affect this activity.

All these results confirmed that mitochondrial fractions were functional for further studies.

3.2. BER glycosylase and endonuclease activity determination

Specific BER glycosylase and endonuclease activities were measured using modified oligonucleotide microarrays designed for the determination of BER capacities in nuclear extracts [20]. As a first step, nuclear BER activities were analyzed to confirm the efficiency of the microarray with fruitfly extracts (Fig. 4A). Most of the eight DNA lesions present on the microarray were detected and excised by these extracts, suggesting the presence of the corresponding specific glycosylases/endonuclease in these samples. However, four main activities were observed in nuclear

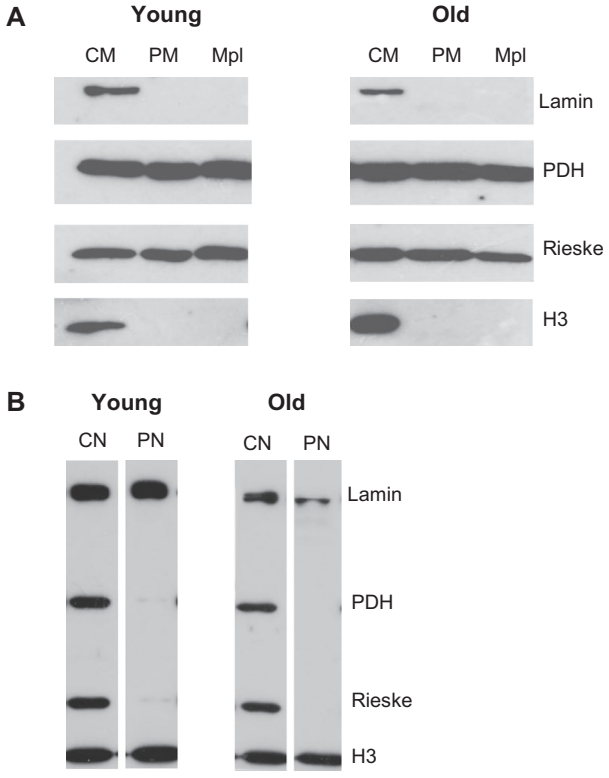

Fig. 2. Western blot analyses of various sub-cellular fractions. Fifteen µg of mitochondrial (A) and nuclear (B) protein fractions were analyzed from young (1 week) and old (8 weeks) fruitflies. Proteins were separated by SDS PAGE, transferred on membrane and analyzed by Western blot. Antibodies directed against mitochondrial components: complex III Rieske subunit and pyruvate dehydrogenase (PDH) or against nuclear components: lamin and histone H3 were used. CM: crude mitochondrial fraction, PM: Percoll gradient purified mitochondria, Mpl: purified mitoplast fraction, CN: crude nuclear fraction and PN: purified nuclear fraction.

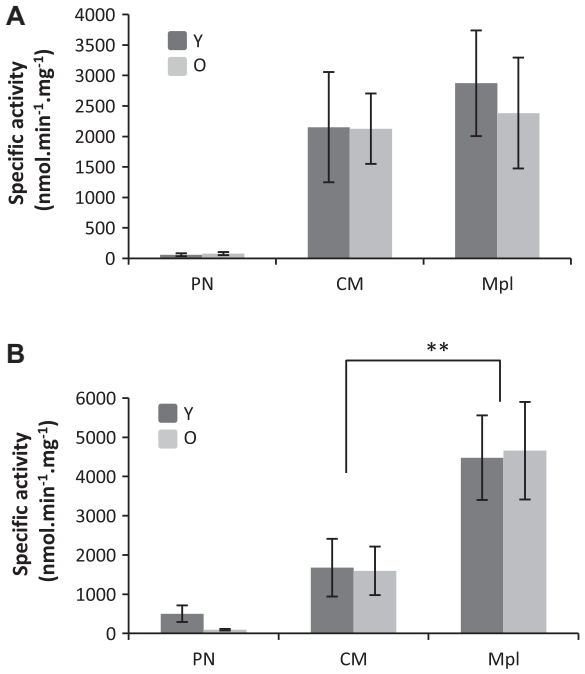

Fig. 3. Enzymatic activity measurements in various sub-cellular fractions. Cytochrome oxidase (A) and citrate synthase (B) activities were measured in various subcellular compartments: purified nuclear fraction (PN), crude mitochondrial fraction (CM) and purified mitoplast fraction (Mpl). Fractions from young (black) and old (gray) fruitflies were analyzed. Values are means of 8 independent experiments and error bars represent the standard deviation (Student's test: $^{**}P < 0.01$).

extracts from young individuals (Fig. 4A, white bars). The endonuclease activity that cleaved THF paired with A with approximately 60% excision rate was predominant. The cleavage rate of Hx, U and A, paired respectively with T, G and 8oxoG was also high (more than 25% excision). Other activities were lower than 10%.

During aging, only Hx-T and A-8oxoG cleaving activities were significantly decreased (68%, $P < 0.05$). Hence, as also for various cell models [21], these modified oligonucleotide microarrays were efficient in determining BER activities in nuclear extracts from *D. melanogaster*.

Mitochondrial fractions, both crude mitochondria (Fig. 4B) and pure mitoplasts (Fig. 4C) from young individuals, were tested in the same conditions. In crude mitochondria samples, four major excision activities were observed with the following efficiency rate order: THF–A (80% excision rate) > U–G > Tg–A > Eth–A. Other activities were not detectable (Hx-T; 8oxoG–C; A-8oxoG and U–A).

The global activity profile for pure mitoplast fraction was quite similar to the one obtained with crude mitochondrial fraction (Fig. 4B and C). The main changes were observed with the excision percentages, which were globally higher in mitoplasts than in crude mitochondria. Moreover, excision of U–A was effective in mitoplast fraction whereas it was almost undetectable in crude mitochondria.

An interesting observation was made regarding the effect of aging (Fig. 4B and C). Two tendencies observed with crude mitochondria were significant with the mitoplasts. A marked increase (2.4-fold, $P < 0.05$) of U–G excision was detected, whereas other activities declined. Aging-associated decrease was significant for THF–A (−18%).

Even though endonuclease activity was high in both nuclear and mitochondrial extracts, these results show that BER activities in mitochondria were different from their nuclear counterpart, and that aging interferes with these capacities.

These results also suggest that crude mitochondrial fractions were pure enough to evaluate specific BER activities from this compartment.

4. Discussion

In our study, drosophila with the ease of genetic, cellular, and cytological approaches was used and constitute a good model for all mitochondrial DNA metabolism (reviewed in Ref. [30]). Aging studies have already been performed and data were collected with this model in our lab [23].

Research on mtDNA repair mechanisms goes back many years, and numerous studies have characterized mitochondrial BER activities, especially glycosylases [10–13]. The most frequent method used to identify and characterize these activities was based on cleavage of a modified oligonucleotide carrying a ^{32}P label [16,17]. However, this technique is not effective enough for investigating the effects of various physiological or pathological conditions on DNA repair capacities. The microarray method used here allows testing simultaneously several glycosylase and endonuclease activities. The sensitivity and specificity of such microarrays have been tested and validated with nuclear BER activities [18–22], but never with mitochondrial extracts.

Mitochondria and aging are strongly related through oxidative stress generation, loss of biochemical functions, mtDNA damages [31], but few studies have been performed on mtDNA repair capacities and their evolution during aging [15,32].

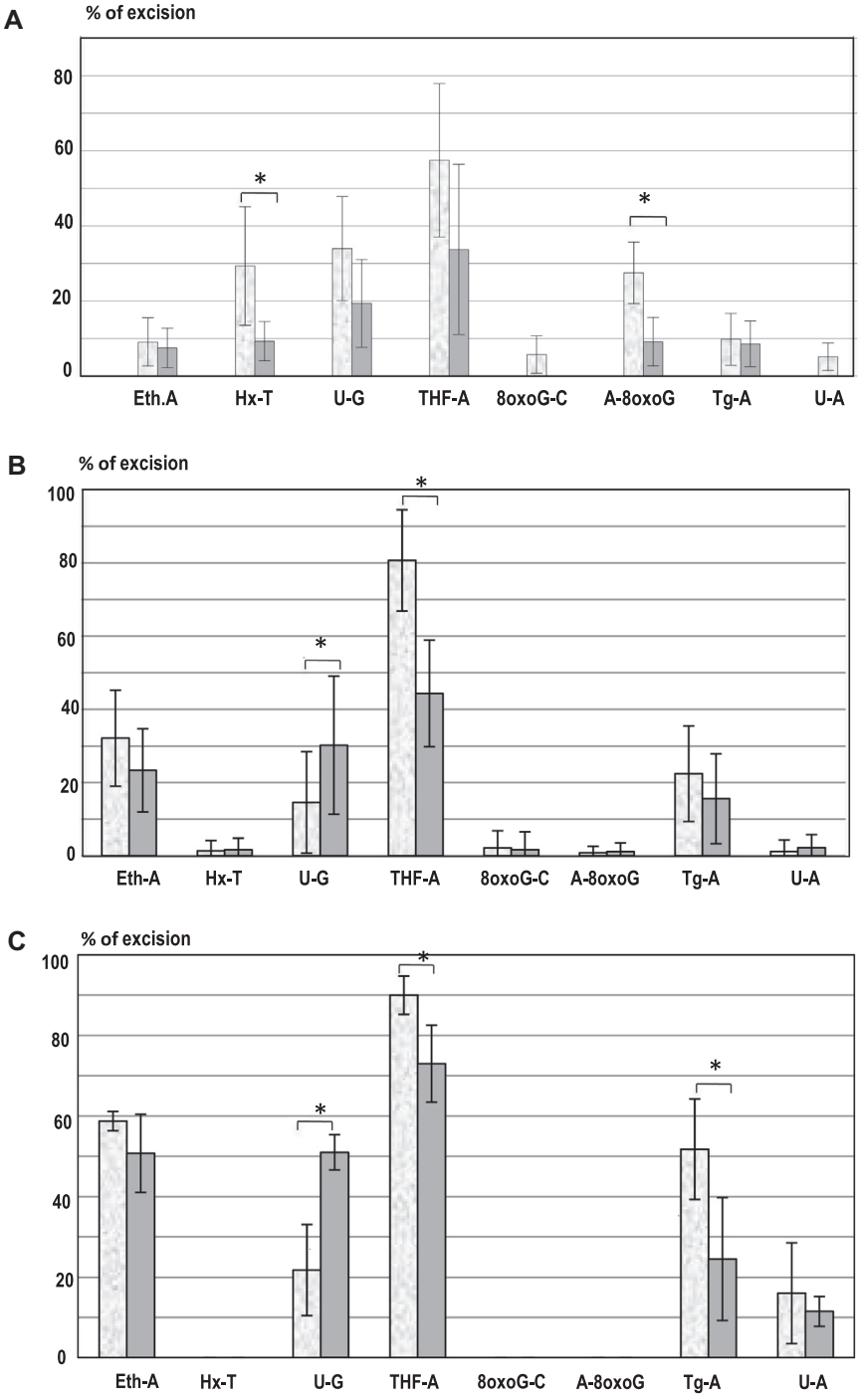

Fig. 4. Cleavage rate measurements using lesion-containing oligonucleotide microarrays. The cleavage rate represents the percentage of released fluorescence at each lesion spot after incubation with purified nuclear fractions (A), crude mitochondrial fraction (B) and purified mitoplasts (C). The different lesion substrates are indicated: ethenoadenine paired with T (Eth–A), hypoxanthine paired with T (Hx-T), uracil paired with G (U–G), tetrahydrofuran paired with A (THF–A), 8oxoguanine paired with C (8oxoG–C), A paired with 8oxoguanine (A-8oxoG), thymine glycol paired with A (Tg–A), uracil paired with A (U–A). Fractions from young (white) and old (black) fruitflies were analyzed. Values are means of 5–8 independent experiments and error bars represent the standard deviation (Student's test: $^{*}P < 0.05$).

High abasic site excision activities in nuclear or mitochondrial fractions were observed. This is the result of apuric/apyrimidic (AP) endonuclease activity such as APE1 or APE2 enzymes in mammals [12,33,34]. APE2 has been shown to be active not only in nuclei, but also in mitochondria. In the fruitfly, RRP1, another endonuclease protein, has been characterized in the nucleus [35], but its mitochondrial location is still unknown. In aging drosophila, this activity is decreased particularly in the mitochondrial fraction. The high decrease observed during the aging process could be related to the increase in mtDNA damages as reported [36]. In another study, an increase was observed in mitochondrial BER capacities [15]. However, this discrepancy compared to our study has to be modulated. In fact, some tissues in mice displayed an increase in mitochondrial BER activities, when other (skeletal muscle) showed a decrease. In our study, the values were the result of changes in whole fly body without specific tissue analyses.

ROS are known to modify adenine and thymine, leading respectively to EthA [37] and Tg [38]. Moreover, A and T are both highly

abundant in mtDNA particularly in the AT-rich sequence [39]. The mitochondrial respiratory chain remains the main source of these damages [1,2]. MPG (methyl-purine glycosylase) [40] or Neil 1, 2 and 3 glycosylases are key enzymes for EthA removal and have been identified in nuclei and detected also in mouse liver mitochondria [41] and human cells [42] but not in drosophila. In our study, aging did not affect this activity compared to Tg removal, which was significantly decreased. Tg presence could lead to stalled replication and mtDNA depletion [38], even if translesion synthesis has been described in human mitochondria depending on polymerase γ but to our knowledge, no data have been described in Drosophila mitochondria [43]. Two main enzymes endonuclease three homolog 1 (NTH1) and RPS3 have been shown to act on Tg in mammals [44,45]. To date, only RPS3 has been identified in Drosophila [46], but its mitochondrial location was only confirmed in human [45].

Uracil presence in DNA as a result of dUMP incorporation during replication or of cytosine oxidative deamination by ROS would lead to U:A pairs or U:G mismatch. Uracil DNA glycosylase (UNG) enzymes correct this damage. In humans, differential splicing of *ung* give a nuclear and a mitochondrial isoforms [44]. But in Drosophila, no ortholog gene has been characterized yet [47]. Moreover, the presence of this abnormal nucleotide in DNA was shown to be very high in larvaes and adults due to strong incorporation of U specifically in Drosophila and lack of repair [48]. Surprisingly in our study, excision of U is significantly increased in aged mitochondria but not in the nuclear fraction. In contrast, this activity was not changed during aging in nucleus and mitochondria from mouse liver [49], but was decreased in human nucleus [21]. We hypothesised that in Drosophila this activity would be developed with aging in mitochondria with a parallel increase of cytosine oxidation in mtDNA.

In our experimental conditions, no 8oxoG removal was detected with mitochondrial extracts either when paired with A or C, compared to nuclear fraction where this activity was observed despite being low, except for A paired with 8oxoG.

In mammals, two different enzymes are characterised with nuclear and mitochondrial locations: MYH for A paired with 8oxoG [44] and 8-oxoguanine DNA glycosylase (OGG1) for 8oxoG paired with C [49,50]. Only OGG1 ortholog has been noticed in *Drosophila* and displayed a nuclear location [51]. However, RPS3 has also been described to be active towards 8oxoG–C excision, without mitochondrial location in fruitfly [46], contrarily to human cells [45]. These two last catalytic activities are not high. Does this suggest that in fruitfly, this base modification would be corrected through an indirect pathway such as A paired with 8oxoG removal as observed in nucleus, or through a still unknown mechanism?

5. Conclusions

Using fruitfly as a model, several mitochondrial DNA glycosylase activities were decreased during aging, suggesting that the corresponding lesions may participate to mtDNA alteration observed during senescence processes. Interestingly, methodology carried out in this analysis was proven to be efficient enough to be a very versatile tool to measure various glycosylase activities in different conditions or sub-cellular compartments. Consequently, these chips could be precious tools for analyzing pathologies involving mtDNA damages.

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