Article

Supplementary Materials: The Potential Protective Effect of the Standardized *Ginkgo biloba* Leaves Extract EGb761 against Contrast-Induced Acute Kidney Toxicity in Rats via Mitigating Renal Tissue Redox Imbalance, Inflammation, Cell Apoptosis and Mitochondrial Damage

Mahmoud M. Farag [1,*], Mariam A. Wagdy [1], Heba E. Ahmed [1], and Amani H. Kazem [2]

[1] Departments of Pharmacology, Medical Research Institute, Alexandria University, Alexandria 21561, Egypt
[2] Departments of Pathology, Medical Research Institute, Alexandria University, Alexandria 21561, Egypt
* Correspondence: mahmoudfarag2012@hotmail.com; Tel.: +20-1006632014
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Assessment of Renal Tissue Mitochondrial DNA (mtDNA) Copy Number Per Cell

- **Principle:**

A quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay is developed to estimate relative levels of mtDNA copy numbers in samples. This approach measures the mtDNA copy number by determining the ratio of PCR amplicons of mitochondrial sequence to that of a single nuclear gene in experimental samples. In this study, after total genomic DNA isolation, we used a specific primer pair for mtDNA and a primer pair for nuclear peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) **(Table S1)** to perform the same number of PCR cycles and calculate the relative mtDNA signal to the nuclear DNA (nDNA) signal. The nuclear target is used to quantify nDNA, and therefore normalization of the mtDNA amount per diploid cell is accomplished [1].

Table S1. Primers for PGC-1α and mtDNA for real time-PCR.

Gene Name	Accession Number		Primer Sequence
PGC-1α	NM_031347.1	F	5′-ATGAATGCAGCGGTCTTAGC-3′
		R	5′-AACAATGGCAGGGTTTGTTC-3′
mtDNA	X14848.1	F	5′-ACACCAAAAGGACGAACCTG-3′
		R	5′- ATGGGGAAGAAGCCCTAGAA-3′

DNA Extraction

Total DNA was isolated from rat kidney tissues using DNeasy mini kit (Qiagen Co., Valencia, CA, USA) according to the manufacturer's instructions.

- **Principle:**

Samples are first lysed using proteinase K. Buffering conditions are adjusted to provide optimal DNA binding conditions and the lysate is loaded onto the DNeasy Mini spin column. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water, ready for use. DNeasy purified DNA has A260/A280 ratios of 1.7–1.9, and absorbance scans show an asymmetric peak at 260 nm confirming high purity.

- **Reagents and materials:**
  - DNeasy Mini Spin Columns (colorless) in 2 mL Collection Tubes
  - Collection Tubes (2 mL)
  - Buffer ATL
  - Buffer AL

- Buffer AW1 (concentrate)
- Buffer AW2 (concentrate)
- Buffer AE
- Proteinase K

Buffer AW1 and Buffer AW2 were supplied as concentrates. Before using it for the first time, we added the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

- **Procedure:**

(1) Tissue samples were removed from storage and 25 mg were used from each sample then cut up into small pieces, and placed in a 1.5 mL microcentrifuge tube.
(2) 180 µL Buffer ATL was added followed by 20 µL proteinase K and mixed well by vortexing.
(3) After that, they were incubated at 56 °C until the tissue was completely lysed.
(4) 200 µL Buffer AL was added to the sample and mixed thoroughly by vortexing. Then 200 µL ethanol (96–100%) was added and mixed again thoroughly by vortexing.
(5) The mixture from step 4 was pipetted (including any precipitate) into the DNeasy Mini spin column placed in a 2 mL collection tube (provided), centrifuged at 6000× *g* (8000 rpm) for 1 min, and the flow-through was discarded.
(6) The DNeasy Mini spin column was placed in a new 2 mL collection tube, 500 µL Buffer AW1 was added, and centrifuged for 1 min at 6000× *g* (8000 rpm). The flow-through was discarded.
(7) The DNeasy Mini spin column was placed in a new 2 mL collection tube, 500 µL Buffer AW2 was added, and centrifuged for 3 min at 20,000× *g* (14,000 rpm) to dry the DNeasy membrane. The flow-through was discarded.
(8) The DNeasy Mini spin column was placed in a clean 2 mL microcentrifuge tube, and 200 µL Buffer AE was pipetted directly onto the DNeasy membrane, incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000× *g* (8000 rpm) to elute.
(9) Elution was repeated once as described in step 8, for maximum DNA yield. (A new microcentrifuge tube was used for the second elution step to prevent dilution of the first eluate)
(10) The suspended DNA sample was stored at −20 °C for subsequent RT-PCR.
(11) The integrity of DNA was confirmed by nanodrop.

RT-qPCR of mitochondrial and nuclear sequences:

RT-qPCR was performed in 25 µL containing 2X SYBR Green PCR Master Mix (Qiagen Co., USA), 500 nM of each primer, and 1 µL of each analyzed DNA sample (Figure S1). The concentration of each analyzed DNA sample was always 50 ng/µL. The thermal cycling conditions are presented in Table S2 [2].

Table S2. Real-Time PCR protocol for mtDNA and nuclear DNA.

	nDNA	**mtDNA**
Initial Denaturation	95 °C for 10 min	
Number of Cycles	45	45
Denaturation	95 °C for 30 s	95 °C for 15 s
Annealing	56 °C for 30 s	60 °C for 15 s
Extension	72 °C for 30 s	72 °C for 30 s
Final Extension	72 °C for 5 min	

Data were collected using Rotor-Gene Q-Pure Detection version 2.1.0 (Qiagen Co., USA).

- **Calculation of mtDNA copy number:**

The relative abundance of the mitochondrial genome was measured as the difference in threshold cycle (ΔCt) values

$$\Delta Ct = Ct_{mtDNA} - Ct_{nuclear}$$

The relative amount of mtDNA to nuclear DNA was calculated by the following equation [3]:

$$R = 2^{-\Delta Ct}$$

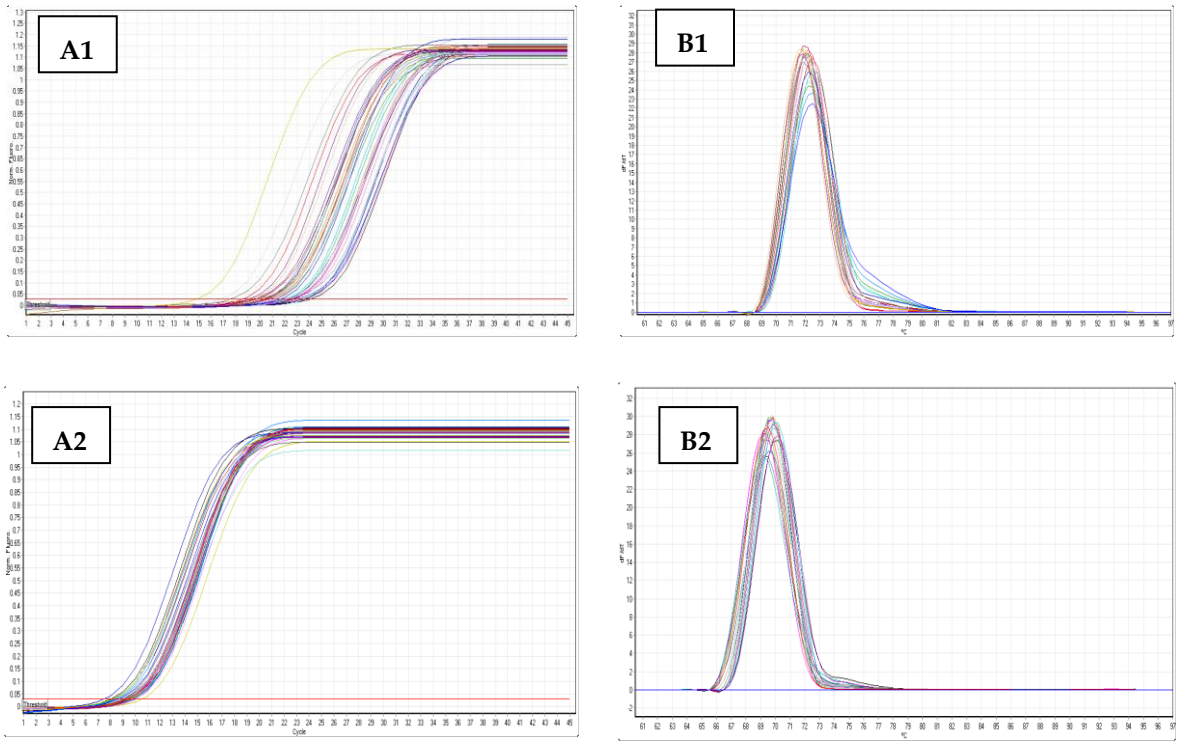

Figure S1. Left: the amplification plots of PCR runs for nuclear PGC-1α (**A1**) and mitochondrial sequence (**A2**) represents the number of cycles on the x-axis and the fluorescence intensity of SYBR Green on the y-axis, the horizontal lines show the threshold line level. **Right**: melting curves of the PCR products of nuclear PGC-1α (**B1**) and mitochondrial sequence (**B2**).

Results of *p* values of the study data analysis for normal distribution using Shapiro-Walk test [4]:

Table S3. *p* values of the present study data analysis using Shapiro-Wilk test.

Experimental Parameters	Control	EGb	CM	EGb + CM
TNF-α (pg/mg protein)	0.337	0.120	0.916	0.532
MDA (nmol/g tissue)	0.553	0.384	0.852	0.594
SOD (U/mg protein)	0.999	0.280	0.266	0.442
GSH (nmol/mg protein)	0.284	0.433	0.178	0.963
GSSG (nmol/mg protein)	0.810	0.774	0.332	0.326
GSH/GSSG ratio	0.528	0.111	0.159	0.432
IL–6 (pg/mg protein)	0.281	0.627	0.622	0.160
IL–1β (pg/mg protein)	0.882	0.757	0.404	0.303
mtDNA (copy number/cell)	0.441	0.069	0.592	0.436
Caspase-3 activity (U/mg protein)	0.961	0.209	0.280	0.863
NGAL (ng/mL)	0.668	0.914	0.964	0.288
Blood urea (mg/dL)	0.318	0.928	0.957	0.191
Serum creatinine (mg/dL)	0.646	0.086	0.089	0.474

$p > 0.05$, in the analysis of all experimental parameters.

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