HIV-1 Tat protein directly induces mitochondrial membrane permeabilization and inactivates cytochrome c oxidase

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The Trans-activator protein (Tat) of human immunodeficiency virus (HIV) is a pleiotropic protein involved in different aspects of AIDS pathogenesis. As a number of viral proteins Tat is suspected to disturb mitochondrial function. We prepared pure synthetic full-length Tat by native chemical ligation (NCL), and Tat peptides, to evaluate their direct effects on isolated mitochondria. Submicromolar doses of synthetic Tat cause a rapid dissipation of the mitochondrial transmembrane potential (ΔΨm) as well as cytochrome c release in mitochondria isolated from mouse liver, heart, and brain. Accordingly, Tat decreases substrate oxidation by mitochondria isolated from these tissues, with oxygen uptake being initially restored by adding cytochrome c. The anion-channel inhibitor 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS) protects isolated mitochondria against Tat-induced mitochondrial membrane permeabilization (MMP), whereas ruthenium red, a ryanodine receptor blocker, does not. Pharmacologic inhibitors of the permeability transition pore, Bax/Bak inhibitors, and recombinant Bcl-2 and Bcl-XL proteins do not reduce Tat-induced MMP. We finally observed that Tat inhibits cytochrome c oxidase (COX) activity in disrupted mitochondria isolated from liver, heart, and brain of both mouse and human samples, making it the first described viral protein to be a potential COX inhibitor.

Mitochondria, the energy-producing organelles of eukaryotes, generate cellular energy in the form of ATP (adenosine triphosphate) by coupling substrates oxidation and the proton gradient established along with electron flow through the electron transfer chain of the respirasome (complexes I, III, and IV) to the ATP synthesis by the F1FO-ATP synthase (complex V).1–3 Mitochondria also play a key role in apoptosis and related forms of cell death.4,5 Mitochondrial fission, inner membrane permeabilization (IMP; leading to mitochondrial transmembrane potential (ΔΨm) loss and matrix cofactor release), rearrangements of mitochondrial lipids, and outer membrane permeabilization (OMP; resulting in the release of, and/or access to, intermembrane space proteins, including cytochrome c) are pivotal events in the apoptotic process.6–8

As a corollary, many viruses have evolved to encode proteins that directly target mitochondria for modulating apoptosis.9,10 The human immunodeficiency virus type 1 (HIV-1) trans-activator of transcription (Tat) protein is an important factor in the HIV-induced pathogenesis of AIDS, contributing to immune dysfunction, Kaposi’s sarcoma, HIV-associated dementia, and cardiomyopathy.11,12 In infected cells, Tat transactivates virus gene transcription and is essential for replication. During acute infection of T cells by HIV, Tat released in the stromal microenvironment of infected cells can bind and/or be efficiently taken up by most cell types.13 Although antiretroviral therapy has proven efficacy to reduce viral load, once proviral DNA is formed, such treatment does not prevent production of early viral proteins Tat, Rev, and Nef.14,15

The outcome of Tat

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activity is dependent on its concentration, the cell types involved, and whether activity is mediated within infected cells or through paracrine-like effect to uninfected bystander cells. Several in vitro studies suggest that Tat may interfere directly or indirectly with mitochondrial functions. For instance, Tat-expressing cell lines have reduced expression of the manganese superoxide dismutase (Mn-SOD), a mitochondrial enzyme that is part of the cellular defense system against oxidative stress. Under low serum conditions, Tat translocation from the nucleus to the mitochondria, correlating with ΔΨ m dissipation, reactive oxygen production, and apoptosis, was also shown in Tat-expressing cell. Tat may also bind tubulin and polymerized microtubules, leading to the alteration of microtubule dynamics and activation of a mitochondria-dependent apoptotic pathway. Using recombinant HIV-1 Tat1-72 protein, Norman et al. reported that Tat can induce a decrease in free mitochondria calcium in primary neurons, and inhibits mitochondrial complexes III and IV in mitochondria isolated from juvenile rat brain. Moreover, transgenic mice expressing Tat selectively in cardiac myocytes present cardiac dysfunction associated with severe mitochondrial damage. Based on these premises, we investigated if pure, biologically active, synthetic Tat may directly interact with mitochondria from different tissues, and which mitochondrial target(s) Tat would possibly hit.

Results

Full-length Tat synthesis. The full-length [1-86] Tat protein is synthesized using the native chemical ligation (NCL) method initially introduced by Dawson et al. The reaction is performed between two fully unprotected peptides, the N-terminal segment having a C-terminus thioester and the C-terminal segment having an N-terminus cysteine residue (Figure 1a). The first step is a trans-thioesterification of the Ca thioester by the thiol function of the Cys residue, and is followed by a spontaneous S to N acyl shift to obtain a native amide bond. The HPLC profile of the reaction products after completion is shown in Figure 1b. The Tat protein is further isolated at a purity above 95% (Figure 1c) and is characterized by mass spectrometry (Figure 1d). The synthesis of this protein by a classical solid-phase protocol has already been reported. The chemical ligation used in the present study represents an alternative and convenient way to obtain a highly purified, well-characterized Tat protein in relatively large amounts (up to 100 mg). This

![Figure 1](https://example.com/figure1.png)
synthetic Tat (sTat) was shown to be fully functional in transactivation assays.25–27 Tat[1-86] induces swelling of isolated mitochondria.

**Figure 2**  Tat-induced swelling in liver isolated mitochondria. (a) Sequence of full-length Tat[1-86] (HIV-1 Lai) and Tat derived peptides. (b) Dose/time response of Tat[1-86]-induced swelling. Isolated mouse liver mitochondria were exposed to full-length Tat at the indicated concentrations and mitochondrial swelling (measured as 90° light scattering at 545 nm) was monitored continuously. (c) Comparative analysis of the effect of Tat-derived peptides on mitochondrial swelling. Isolated mouse liver mitochondria were exposed to the indicated concentrations of Tat-derived peptides. Mitochondrial swelling was monitored for 30 min. Percentages of mitochondrial swelling were calculated as described under Materials and Methods. Data are means (± S.D.) of three independent experiments. (d) Evaluation of PTP-related inhibitors on mitochondrial swelling. Liver mitochondria were exposed to Tat[1-86] (0.3 μM; 30 min) in the presence or absence (Co.) of the following compounds (added 5 min before Tat): cyclosporin A (CsA; 30 μM), ADP (1 mM), bongkrekic acid (BA; 50 μM), Bcl-2 (400 nM), Bcl-XL (400 nM), or DIDS (5 μM). Histograms represent mean values (± S.D.) of five independent experiments. *P < 0.05. (e) Time course follow-up of absorbance characteristics of mouse isolated liver mitochondria in the absence of Co. and the presence of either Tat[1-86] or Tat + 5 μM DIDS. Excess of DIDS was removed by centrifugation before Tat was added at the final concentration of 0.3 μM. (f) Ultrastructure of Tat-treated mitochondria. Representative electron micrographs of isolated liver mitochondria treated or not (Co.) with Tat[1-86] (30 min; 0.15 μM) and optionally pre-treated with DIDS (5 μM; 1 min before Tat addition)
positive control was defined by the addition of 50 μM CaCl₂ and mitochondrial swelling (measured as 90° light scattering at 545 nm) was monitored for 30 min. When indicated, mitochondria were preexposed for 5 min in the presence or absence of the following compounds: cyclosporin A (CsA; 30 μM), ADP (1 mM), bongkrekic acid (BA; 50 μM), Bcl-2 (400 nM), or DIDS (5 μM). Then, mitochondria were incubated with Tat[1-86] (0.3 μM; 30 min). Percentages of mitochondrial swelling (left panel) were calculated as described under Materials and Methods. Positive control was defined by the addition of 50 μM CaCl₂. Histograms represent mean values (± S.E.M) of three independent experiments.

Characterization of Tat-induced ∆Ψₚₚ and cytochrome c release in isolated mitochondria. Real-time and fixed-time flow cytometry analysis of ∆Ψₚₚ indicates that full-length Tat induces a rapid ∆Ψₚₚ loss in liver mitochondria, heart mitochondria, and brain mitochondria as well (Figures 4a and b). Tat-induced ∆Ψₚₚ loss is inhibited by DIDS in mitochondria isolated from these three tissues (Figure 4b). Contrary to DIDS, PTP inhibitors and recombinant Bcl-2 are unable to hamper Tat-induced ∆Ψₚₚ loss (Figure 4c). We also found that Tat-induced ∆Ψₚₚ loss is associated with DIDS-sensitive cytochrome c release (Figures 3d and e). As mitochondria isolated from heart and liver do not express Bax but do express low and high quantities of Bak, respectively (Supplementary Figure S3), we also investigated whether Bax-inhibiting peptide (BIP) or Bax channel inhibitor (BCB; which inhibits Bak and Bak oligomerization in the mitochondrial outer membrane⁵) might affect Tat-induced cytochrome c release (Figures 4f and g). Neither BIP nor BCB could modify Tat-induced cytochrome c release. Taken together with the absence of the effect of Bcl-2 (Figures 2d and 4c), one can suggest that Tat-induced MMP might be independent of the canonic Bax/Bak-mediated mitochondrial permeabilization pathway.

Tat inhibits substrate oxidation in mitochondria isolated from heart, brain, and liver. Compared with untreated organelles (Figure 5a, traces a, e, g), mitochondrial preincubated with Tat[1-86] (Figure 5a, traces b, f, h) exhibit a severe deficiency in succinate oxidation. Adding cytochrome c to Tat-treated mitochondria oxidizing succinate does not stimulate the rate of oxygen uptake (Figure 5a, traces b, f, h), suggesting that Tat inhibits respiratory chain function by an additional mechanism. Noticeably, DIDS partly prevents Tat-induced succinate oxidation defect and permits a full restoration of oxygen consumption by exogenous cytochrome c (Figure 5a, trace c). In contrast, RYR appears not to be implicated as we observed that respiratory control ratios (RCRs) measured using succinate as a substrate in heart mitochondria did not differ in the absence (RCR: 2.7) or presence (RCR: 2.8) of 1 μM ruthenium red (Supplementary Figure S4). Tat-induced inhibition of substrate oxidation is dose dependent (Figure 5b). Interestingly, up to 5 μM Tat, the addition of exogenous cytochrome c reverses Tat-induced inhibition of substrate oxidation (Figure 5b). This suggests that Tat-induced OMP and IMP may be two independent/ successive events.

Full length HIV-1 Tat is a cytochrome c oxidase (COX) inhibitor. The activity assays of complexes I–IV in brain mouse homogenate show that complex IV (but not electron transfer complexes I– III) is strongly inhibited (>95%) by 1 μM Tat[1-86] (Table 1). To determine if full-length Tat inhibits COX activity in liver mitochondria, we evaluated the effects of Tat[1-86] on the ability of COX to oxidize exogenous cytochrome c in permeabilized organelles.⁴ In order to avoid medium-induced artifacts, experiments were performed in three different media classically used for swelling, respirometry, and COX enzymatic activity evaluations, respectively. In lauryl maltoside-treated liver mitochondria, Tat[1-86] strongly inhibits cytochrome c oxidation whatever the medium considered (Figure 6a, panel 1), and this effect is not prevented by DIDS pretreatment (Figure 6b, panel 2). In contrast, Vpr52-96, another MMP-inducing HIV-related peptide, does not induce COX inhibition (Figure 6a, panel 3). Shorter Tat-derived peptides do not (Tat[44-61] and Tat[61-86]), or only poorly (Tat[30-86]), inhibit mitochondrial COX (Figure 6a, panel 4).

COX inhibition by full-length Tat is not tissue specific as a dose-dependent COX inhibition is also observed in (lauryl maltoside) permeabilized mitochondria isolated from heart and brain (Figure 6b). We next determined the...
kinetics properties of COX in liver mitochondria under our experimental conditions ($V_{\text{max}} = 73 \pm 8$/s; $K_m (\text{red cyt } c) = 1.95 (\pm 0.8)$ $\mu$M). Tat addition to isolated liver mitochondria reduces $V_{\text{max}}$ ($V_{\text{max}} [\text{Tat}] = 35 (\pm 4)$$/s) without any effect on $K_m (\text{red cyt } c)$, suggesting that Tat does not directly compete with cytochrome $c$ (Figure 6c). Finally, Tat[1-86] is able to inhibit COX activity in homogenates from various human tissues including liver, heart, brain, and skeletal muscle (Supplementary Figure S5).

**Discussion**

In the present study, we have investigated the potential direct effect of synthetic Tat protein (from HIV-1 Lai isolate; clade B) on mitochondria that could trigger pathogenic events. Based on the evidences obtained with isolated liver mitochondria (liver, heart, and brain), it appears that the HIV-1-encoded Tat[1-86] protein directly interacts with mitochondrial membranes, triggering PTP-independent $\Delta \Psi_m$ loss and cytochrome $c$ release. Under our experimental conditions, Tat also induces mitochondrial swelling in liver and heart mitochondria. All these Tat-induced MMP events are prevented by the general anion channel blocker DIDS but not by PTP inhibitors or Bax/Bak inhibitors.

We also report for the first time a severe decrease of succinate oxidation upon Tat addition to intact mitochondria from heart, brain, and liver mice. Interestingly, when Tat is added to these mitochondria after disruption, or to various human homogenates (liver, heart, brain, and skeletal muscle), a severe and specific COX inhibition is observed, whereas other mitochondrial respiratory chain complexes (I–III) are not affected. A previous report showed that recombinant HIV-1 Tat[1-72] could induce a slight decrease in rat brain complex III and IV activities.21 Our results do not confirm any effect of
HIV-1 Tat targets mitochondrial functions

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Figure 5 Oxidative properties of purified mitochondria exposed to Tat. (a) Oxygen consumption upon addition of the indicated reagents. Trace a: liver mitochondria (no pretreatment). Trace b: Liver mitochondria pretreated for 3 min with 10 μM Tat. Trace c: liver mitochondria pretreated 1 min with 10 μM DIDS, then 3 min with 10 μM Tat. Trace d: liver mitochondria pretreated with 10 μM DIDS only. Trace e: heart mitochondria (no pretreatment). Trace f: heart mitochondria pretreated for 3 min with 10 μM Tat. Trace g: brain mitochondria (no pretreatment). Trace h: brain mitochondria (no pretreatment). (b) Influence of Tat concentration on oxidative activities of liver, heart, and brain mitochondria. Oxygen consumption by purified mitochondria was measured after addition of succinate (as in (a)). Mitochondria were treated 3 min with the indicated concentrations of Tat. Then, oxygen uptake by purified mitochondria was measured after addition of succinate (as in (a)) in the absence (gray bars) or presence of (black bars) exogenous cytochrome c. Histograms represent % mean respiratory activity (n = 3; variability was < 5%). Calculations are as described under Materials and Methods.

Table 1 Tat effects on respiratory chain complex activities

| Brain homogenate | Malonate-sensitive SCCR (CII–III) | Antimycin-sensitive NCCR (CI+III) | Rotenone-sensitive QCCR (CIII) | COX (CIV) |
|------------------|----------------------------------|----------------------------------|--------------------------------|--------|
|                  | (nmol/min per mg protein)        |                                  |                                |        |
| Co.              |                                  | 16                               | 48                             | 20     | 63     |
| Tat 1 μM         | 15                               | 45                               | 20                             | 2      | 63     |
| Buffer (5 μl)    | —                                | —                                | —                              | —      | 63     |

Abbreviations: SCCR, succinate cytochrome c reductase; QCCR, quinol cytochrome c reductase; NCCR, NADH cytochrome c reductase.

The data are means of triplicate experiments. Variability was < 5%.

Activities of the various segments of the respiratory chain (succinate cytochrome c reductase and NADH cytochrome c reductase) were determined spectrophotometrically as described in Materials and Methods.

mitochondria, and favors apoptosis.19 Although it is plausible that such mechanism could occur in other tissues, we show clearly that the direct effect of Tat on isolated mitochondria does not require such modification as the Tat effects we have observed are reproduced with Tat[30–86].

A number of COX inhibitors have been previously described, including the well-known sodium azide, cyanide, carbon monoxide, nitric oxide,36–38 D-2-hydroxyglutaric acid,39 4-hydroxy-2-nonenal,40 cephalosporins,41 or Alzheimer’s amyloid precursor protein 695.42 However, HIV Tat is the first viral protein inhibiting COX. The respirometry experiments show that Tat effect with concentrations up to 5 μM can be essentially counterbalanced by exogenous cytochrome c addition. This indicates that the decrease of oxygen uptake observed under our experimental conditions is initially because of OMP and loss of cytochrome c rather than COX inhibition. Taken together, these experiments suggest that when Tat reaches mitochondria (at the doses studied), it permeabilizes outer membrane to cytochrome c but does not initially target COX. Under our in vitro conditions, inhibition of this latter complex by Tat only happens after disruption of mitochondrial membranes. Accordingly, in situ studies of immunohistochemistry-based detection of COX activity in Tat-treated lymphocytes and neurons indicate that, when added at sublethal concentrations, Tat does not affect COX activity in intact cells. In the context of HIV infection, long-term chronic exposures to Tat might lead to a progressive and local mitochondrial accumulation and result in COX inhibition in the absence of, or before, OMP.

HIV infection is associated with profound cellular alterations including immune dysfunctions and neurological and cardiac complications.43–45 HIV Tat has been involved in many aspects of AIDS pathogenesis,43–45 including apoptosis of different cell types.12,46–48 Hence, Tat cytotoxicity may be (at least in part) related to direct MMP induction, possibly triggering a cytochrome c-dependent apoptotic pathway. Other HIV-1 proteins, Vpr, Env, and PR, may also directly or indirectly affect mitochondrial function; Vpr via a direct PTP interaction,29 PR by cleaving procaspase-8 and/or Bcl-2,49,50 and Env by a cell-to-cell-mediated signaling pathway leading to Bax activation.51–54 This hints at the possibility that several apoptogenic HIV-1 proteins – Vpr, Tat, Env, and PR – cooperate at the mitochondrial level, contributing to
HIV-related cell damage in lymphocytes, neurons, and cardiomyocytes.

Materials and Methods

Animals. Mice were housed with a 12-h light/dark cycle. Free access to a standard laboratory chow diet and drinking water was provided. Experimental procedures were conducted according to the European Community guidelines for the care and use of experimental animals. Mice experimentation conducted at Theraptosis were approved by the local animal ethical committee (Biotech, Romainville, France). Mice experimentation conducted at Inserm U676 was approved by the animal ethical institutional review committee, according to the INSERM guidelines, and was carried out in accordance with the European Community guidelines for the care and use of experimental animals.

Mitochondria isolation and purification. Liver and heart mitochondria were isolated from 4- to 6-week-old BALB/c mice (IFFA CREDO, Saint-Germain sur l’Arbresle, France). Liver mitochondria were prepared by standard differential centrifugations followed by Percoll purification as described previously. Mice hearts were minced and homogenized with a Dounce homogenizer in buffer containing 300 mM sucrose, 5 mM TES (N-tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid) pH 7.2, 0.2 mM EGTA, and 1 mg/ml BSA. The suspension was centrifuged at 800 g for 10 min and the resulting supernatant at 10 000 g for 10 min at 4 C. The pellet was resuspended in homogenization buffer before to be layered on a three-phase percoll density gradient. After centrifugation (for 10 min at 8740 g), mitochondria were collected from the lower interface and washed in homogenization buffer by centrifugation at 10 000 g (10 min). Brain mitochondria were isolated according to previously described protocols and used in spectrophotometry, spectrofluorimetry, and respirometry assays. We did not find significant differences in most read-outs when using the alternative purification protocols as compared with the percoll density gradient-based purifications. Only flow cytometry-based assays strictly requested gradient-based purification.

Human tissue. Human liver, brain, and heart homogenates were prepared from 5 to 10 mg post-mortem tissues for diagnostic purpose with informed consents. Microaliquots (10–30 µl left after diagnostic investigation) used in this study presented normal activity of the respiratory chain complexes.

Figure 6 Cytochrome c oxidase inhibition by Tat protein and Tat-derived peptides in permeabilized mitochondria. (a) Assay of cytochrome c oxidase (COX) activity in detergent-treated (2.5 mM dodecylmaltoside) mouse liver mitochondria. Part 1: Inhibition of cytochrome c oxidation in permeabilized mitochondria induced by the addition of 1 µM Tat, versus untreated mitochondria (Co.). Experiments were performed in three distinct media, namely COX (M1), swelling (M2), and electrode (M3) media. Part 2: Absence of protective effect of 5 µM DIDS against COX inhibition by 1 µM Tat[1-86]. Part 3: Similar experiment with 1 µM of the negative control Vpr 52-96. Part 4: Effects of 1 µM Tat[1-86] and Tat-derived peptides (30–86, 44–61 and 61–86) on COX activity (n = 3). (b) Inhibition of COX activity in mouse liver, brain, and heart mitochondria by increasing concentration of Tat[1-86]. DIDS was added at the indicated concentration. (c) Km (red Cyt c) calculation in the presence or not of full-length Tat.
Synthesis of full-length Tat and Tat peptides. Tat-[1-86] protein from HIV-1 Lai strain was prepared using the NCL method introduced by Dawson et al., as described in Supplementary Materials and Methods. Other Tat fragments used in this study, namely Tat-[30-86], Tat-[48-86], Tat-[61-86], Tat-[30-61], and Tat-[44-61], were assembled using solid-phase FMoc chemistry, purified, and analyzed as described for the Tat-[22-86] fragment.

Reagents. ADP, BA, ruthenium red, DIDS, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma (Saint Quentin Fallavier, France), CSF from BICMOL Research Laboratories (Le Penny-en-Yvelines, France), and the recombinant proteins BcI-2 and BcI-xl from Oncogene Research products (Oncogene Research Products, Merck, WVR International, Fontenay-sous-Bois, France). BCB (6)-1-(3,6-dibromocarbazol-9-yl)-3-piperazin-1-yl-propan-2-ol was purchased from Calbiochem (San Diego, CA, USA) and BIP-VS (H-Val-Pro-Met-Leu-Lys-OH cat. no. 196810) was purchased from Calbiochem (Nottingham, UK).

Detection of large amplitude swelling and \( \Delta \Psi m \) loss. Mitochondria were resuspended in a buffer, referred as the swelling buffer, containing 0.2 M sucrose, 5 mM succinate, 10 mM MOPS (pH 7.4) made from NaCl, 2 mM Potassium, and 15 mM EGTA. Large amplitude swelling was determined by measuring absorbance at 545 nm (\( A_{545} \)). Percentages of specific swelling were calculated as follows: (\( A_{545} \) - \( A_{545} \)) \times 100/\( A_{545} \). Where \( A_{545} \) - \( A_{545} \) correspond to the absorbance value obtained for CaCI2-treated, reagent-treated, and pretreated mitochondria respectively. \( \Delta \Psi m \) loss was assessed by 5,5',6,6'-tetrachloro-1,3,3'-tetraethyl-benzimidazolylcarboxylic acid (JC-1; Molecular Probes, Saint Aubin, France) incorporation followed by fixed- and real-time flow cytometry analysis. Alternatively, mitochondrial swelling and \( \Delta \Psi m \) were analyzed by spectrophotometry and spectrofluorimetry in 96-well plates (2 mM ADP, BA, ruthenium red, DIDS, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from SIGMA (Saint Quentin Fallavier, France). The relative respiratory activity of mitochondria treated by Tat was calculated as follows: respiratory activity of Tat-treated and control mitochondria, respectively.

Electron microscopy. Isolated mitochondria were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 3 h at 4°C. After several washes with this buffer, specimens were post-fixed with 1% osmium tetroxide containing 1.5% potassium cyanoplatinate, dehydrated in gradual ethanol (30→100%), and embedded in Epon. Thin sections (70 nm) were collected onto 200 mesh copper grids, and counterstained with uranyl acetate and lead citrate before examination with a Philips CM12 transmission electron microscope (Philips Research, Eindhoven, The Netherlands) at 80 kV.

Statistical analysis. Data obtained on isolated mitochondria were analyzed using Student’s t-test for all pairwise comparisons of mean responses among the different treatments or conditions tested. Results are presented as the mean ± S.D. for replicate experiments. Differences were considered significant when \( P < 0.05 \).
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