Elevated levels of macrophage migration inhibitory factor (MIF) in the plasma of HIV-1-infected patients and in HIV-1-infected cell cultures: A relevant role on viral replication

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A B S T R A C T
The cytokine macrophage migration inhibitory factor (MIF) is involved in the pathogenesis of inflammatory and infectious diseases, however its role in HIV-1 infection is unknown. Here we show that HIV-1-infected patients present elevated plasma levels of MIF, that HIV-1-infected peripheral blood mononuclear cells (PBMCs) release a greater amount of MIF, and that the HIV-1 envelope glycoprotein gp120 induces MIF secretion from uninfected PBMCs. The HIV-1 replication in PBMCs declines when these cells are treated with anti-MIF antibodies, and exposure of HIV-1-infected cells to the ABC-transporter inhibitor probenecid results in inhibition of MIF secretion. The addition of recombinant MIF (rhMIF) to HIV-1-infected PBMCs enhances viral replication of CCR5- or CXCR4-tropic HIV-1 isolates. Using a T CD4+ cell lineage containing an HIV long terminal repeats (LTR)-Luciferase construct, we detected that rhMIF promotes transcription from HIV-1 LTR. Our results show that HIV-1 induces MIF secretion and suggest that MIF influences the HIV-1 biology through activation of HIV-1 LTR.

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I N T R O D U C T I O N
Infection by the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the acquired immunodeficiency syndrome (AIDS), is a worldwide disease characterized by a persistent viral replication in lymphoid tissues, leading to a profound immunosuppression that can culminate in higher susceptibility to opportunistic infections, tumors and central nervous system degeneration (Stevenson, 2003). HIV-1 infects and replicates in CD4+ lymphocytes and monocyte/macrophages, using the CD4 molecule (Dalgleish et al., 1984) and the chemokine receptors CXCR4 (Feng et al., 1996) or CCR5 (Deng et al., 1996) to penetrate into host cells. A variety of conditions have been associated with increases in viral load and viral persistence in HIV-1-infected individuals, such as infections (Orenstein et al., 1997; Toossi et al., 1993; Wahl et al., 1999) and immune activation (Ortigao-de-Sampaio et al., 1998; Stanley et al., 1996). Immune activation in HIV-1-infected patients has been recently related to microbial translocation from the gut to circulation secondary to an increased intestinal permeability associated with destruction of the gut lymphoid tissue (GALT) (Brenchley et al., 2006a; Brenchley et al., 2006b; Lee et al., 2009). Therefore, activation of the immune cells by microbial products (such as LPS) and interaction of HIV-1-infected cells with host molecules can modulate viral replication and influence the disease progression.

One of the most complex aspects of AIDS pathogenesis is the dynamic interplay between HIV-1 and host molecules (Hladik and McElrath, 2008; Neil et al., 2008). In this context, cytokines and other inflammatory mediators play a pivotal role in the viral replicative cycle (Alfano et al., 2008; Kedzierska and Crowe, 2001), and a number of studies support the influence of these molecules over both the course of the disease and HIV-1 biology (Alfano et al., 2008; Copeland, 2005; Kedzierska and Crowe, 2001). Pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-6, are considered to up-regulate HIV-1 replication, whereas certain anti-inflammatory cytokines, such as type 1 Interferon (IFN), IL-10 and IL-27, may diminish viral replication (Alfano et al., 2008; Kedzierska and Crowe, 2001). Some cytokines are also capable of interfering in the HIV-1 replication by means of modulation of cell restriction factors to the virus. For example, production or activation of the proteins of the APOBEC family and recently described Tetherin are regulated by IL-2,
IL-7, IL-15 and IFN-α (Neil et al., 2008; Stopak et al., 2007; Varthakavi et al., 2008).

Macrophage migration inhibitory factor (MIF) is an upstream activator of innate immunity, it is present pre-formed in many cell types and it is endowed with both enzymatic and receptor binding properties (Bernhagen et al., 2007; Calandra and Roger, 2003; Leng et al., 2003). MIF lacks a signal sequence and it is secreted by a specialized pathway that is dependent of ATP-binding cassette (ABC) transporters, specifically from the family ABCA1 (Flieger et al., 2003). Another unique characteristic of MIF is the fact that it binds and activates a multi-component receptor complex comprising CD74, CD44, and the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007; Calandra and Roger, 2003; Shi et al., 2006). MIF promotes the release of a number of pro-inflammatory cytokines, such as TNF-α, IL-6 and Prostaglandin E₂ (PGE₂) (Calandra and Roger, 2003).

Elevated serum levels of MIF have been detected in many infectious and inflammatory diseases, such as rheumatoid arthritis, atherosclerosis (Ayoub et al., 2008), sepsis (Bozza et al., 2004; Bozza et al., 1999; Emonts et al., 2007), West Nile Virus (Arjona et al., 2007) and others (Calandra and Roger, 2003; Hoi et al., 2007). Moreover, MIF has been implicated in the worsening of some pathological conditions, and neutralization of this cytokine ameliorates the disease clinical course (Anano et al., 2007; Bozza et al., 2004; Calandra et al., 2000; Chen et al., 2006; Gando et al., 2007; Ichiyama et al., 2004; Meazza et al., 2002).

Since there are no studies addressing the role of MIF in HIV-1 infection and its ability to modulate HIV-1 replication, we measured the levels of this cytokine in the plasma of HIV-1-infected patients and investigated whether it could influence HIV-1 replication in human primary peripheral blood mononuclear cells (PBMCs). Here, we show for the first time that plasma levels of MIF are elevated in HIV-1-infected individuals, PBMCs release MIF upon HIV-1 infection, and that MIF secretion contributes to viral replication.

Results

HIV-1-infected patients present high plasma levels of MIF

HIV-1 infection increases the secretion of many cytokines in vitro and in vivo (Kedzierska and Crowe, 2001), and enhancement of MIF production has been detected either in the sera of patients or at the site of some inflammatory and infectious diseases (Awandare et al., 2007; He et al., 2006). Also, it is already known that viral infections, such as the dengue and West Nile virus infection, can up-regulate serum levels of MIF (Arjona et al., 2007; Chen et al., 2006). Thus, we hypothesized that a similar phenomenon could be occurring in HIV-1-infected individuals, and we first examined whether these patients would present higher plasma levels of MIF relative to uninfected individuals. We selected a group of HIV-1-infected individuals (n = 30) and a control group comprised of healthy volunteers (n = 10). Plasma was collected from these individuals and the levels of MIF were investigated by ELISA. Plasma from healthy volunteers (control plasmas; HIV-1 neg) were assessed for MIF concentrations by ELISA. Bars represent means ± SEM of 10 healthy volunteers (HIV-1 neg) or 30 HIV-1-infected patients (HIV-1 pos). *p < 0.0001.

MIF is secreted by primary PBMCs infected with HIV-1 in vitro

Based on the findings that some viral infections can up-regulate MIF secretion in vitro, such as West Nile virus, human Cytomegalovirus (CMV) and Influenza A infections (Arjona et al., 2007; Arndt et al., 2002; Bacher et al., 2002), and in order to understand if HIV-1 infection itself could be contributing for these higher levels of MIF in the plasma of infected patients, we infected human primary PBMCs and macrophages and examined whether HIV-1 infection would induce these cells to secrete MIF. PBMCs were infected with either an R5-tropic or an X4-tropic HIV-1 isolate and MIF secretion and viral replication were evaluated 4 and 7 days after infection. We detected that, infection with HIV-1 R5-tropic isolate doubled the release of MIF by these cells relative to MIF secretion by PBMCs (Fig. 2A and B). Of note, there was no significant increase in secreted MIF at 4 days of infection (data not shown), suggesting that MIF release may follow a different kinetics to virus replication in PBMC, since HIV-1 replication usually peaks about 7 days after infection of these cells. The increment in MIF secretion by HIV-1-infected PBMC is not restricted by the phenotype of the HIV-1 isolate, as evidenced by the fact that the infection by an X4-tropic strain also increased MIF secretion, as measured 7 days after infection (Fig. 2C). We also measured MIF secretion by HIV-1-infected macrophage-derived macrophages and, curiously, HIV-1 infection of macrophages did not alter the secretion of basal levels of MIF (Fig. 2D), as observed in two time points (7 and 14 days after infection), suggesting a phenomenon restricted to PBMCs.

HIV-1 gp120 also induces MIF secretion by PBMC

Based on our findings that the increment of MIF secretion was significant 7 days after infection, at the same time point when elevated amounts of HIV-1 production are also detected, we hypothesized that the HIV-1 virion per se, or HIV-1 components could induce direct secretion of MIF through interaction with infected or uninfected PBMCs in culture. In fact, it has been described that viral components can accumulate in cultures of HIV-1 infected PBMCs, such as gp120 (Zhang et al., 1997). Based on the ability of HIV-1 envelope-derived gp120 protein to trigger the secretion of a variety of cytokines and that gp120 can be detected in serum of HIV-1+ patients (Capobianchi, 1996; Klasse and Moore, 2004), we exposed uninfected PBMCs to a concentration of soluble recombinant Bal gp120 (r gp120) protein previously known to induce cytokine secretion (5 μg/mL) for 16 h (Schols and De Clercq, 1996). We observed that r gp120 promoted MIF secretion by treated PBMC (Fig. 2E) at approximately 50% more than untreated cells, suggesting that viral components can contribute to the enhanced MIF release by HIV-1-infected PBMC cultures.

HIV-1 infection stimulates MIF release through ABCA1 transporters

The release of MIF upon stimulation by microbial components such as lipopolysaccharide (LPS) has been shown to be dependent on the ABCA1-family of the ATP-binding cassette (ABC) transporters (Flieger et al., 2003). Thus, it was of interest to examine whether ABC transporters mediate the MIF release promoted by an infectious agent such as HIV-1. Accordingly, cells were pre-treated with probenecid (10 μM,
for 45 min), infected with HIV-1, and probenecid immediately re-applied to infected cells. MIF release was measured at day 7 post-infection. Probenecid treatment resulted in a reduction of MIF secretion secondary to HIV-1 infection (Fig. 3), which is consistent with the notion that the HIV-1-induced MIF release is dependent of ABCA1 transporters.

**Immunoneutralization of secreted MIF diminishes HIV-1 replication**

Based on the preceeding result that HIV-1 infection induces MIF secretion and to provide evidence for the role of MIF in HIV-1 replication, HIV-1-infected PBMCs were treated with neutralizing anti-MIF polyclonal antibodies immediately after infection, and viral
replication was evaluated 7 days after infection. MIF immunoneutralization reduced HIV-1 replication by approximately 40% (Fig. 4), indicating that MIF inhibition down-regulates HIV-1 replication. This result suggests that MIF, similar to other pro-inflammatory cytokines, can promote HIV-1 replication in primary HIV-1-infected cells (Kinter et al., 2000).

Addition of rhMIF to HIV-1-infected PBMCs enhances viral replication

Since immunoneutralization of MIF decreased HIV-1 replication, we next asked whether the addition of rhMIF could augment viral replication. Based on the observation that MIF can be detected at concentrations reaching 25 ng/mL in plasma of HIV-1-infected patients, we added rhMIF (obtained as described in Bernhagen et al., 1994) to HIV-1-infected PBMCs just after the infection, and measured viral replication after 7 days. We found that MIF significantly enhanced HIV-1 replication of a R5-tropic isolate at doses of 12 and 25 ng/mL, when compared to untreated HIV-1 infected cells (Fig. 5A). MIF also exacerbated the viral replication of an X4-tropic isolate (Fig. 5B), indicating that the ability of MIF to enhance viral replication is independent of viral phenotype.

The rhMIF support HIV-1 transcription from LTR

Next, we addressed whether MIF could stimulate viral replication via induction of HIV-1 transcription from LTR. For this purpose, we added rhMIF to cultures of a Jurkat CD4+ T derivative cell line (1G5)
containing a stably integrated HIV-LTR-Luciferase construct. In this system, Luciferase expression is controlled by transcription from LTR, and a quantitative analysis of transcription is possible through luminescence signal emitted by Luciferase (Aguilar-Cordova et al., 1994). As depicted in Fig. 6, exogenous treatment with 25 ng/mL of rhMIF increased the transcription from HIV-1 LTR, suggesting that MIF drives a biochemical signaling pathway which culminates on HIV-1 transcription. As positive controls, we used PHA stimulation, as well as HIV1 Tat protein, once this cell lineage was selected for high responsiveness to these molecules. Taken together, these results indicate that rhMIF augments HIV-1 replication via induction of direct HIV-1 transcription.

Discussion

Macrophage migration inhibitory factor (MIF) exhibits a variety of physiological activities, including enzymatic activity and the property of being released by the pituitary upon stress situations (Bernhagen et al., 1994; Calandra and Roger, 2003; Larson and Horak, 2006). This cytokine has also been implicated in many inflammatory and infectious diseases, such as sepsis and rheumatoid arthritis, to name a few, and in many of these conditions elevated levels of MIF in the circulation were found, and MIF neutralization led to amelioration of the targeted pathology (Javed and Zhao, 2008).

HIV-1 infection is known to be profoundly regulated by immune molecules such as cytokines and others soluble factors, that have been described to modulate HIV-1 replication, and which levels are commonly found altered in plasma of HIV-1-infected patients. Therefore, cytokines have been considered pivotal players in the pathogenesis of HIV-1 infection: they both balance the immune response and promote or inhibit virus replication (Alfano et al., 2008; Kedzierska and Crowe, 2001).

Although the role of cytokines in HIV-1 infection and pathogenesis has largely been explored, there are no reports, so far, about the participation of MIF in this condition. Because MIF can activate several signaling pathways which can ultimately facilitate HIV-1 replication, such as activation of the transcription factor NF-κB, PGE2 induction and TNF-α secretion (Amin et al., 2006; Donnelly et al., 1997; Mitchell et al., 2002), we decided to analyze whether MIF could also be involved in HIV-1 biology, uncovering it as an upstream factor contributing to the delicate balance of the interaction between host and pathogen in the course of this infection. Our findings constitute the first report that MIF is present in elevated concentrations in the plasma of HIV-1-infected individuals and that MIF is secreted by HIV-1-infected cells or by an HIV-1 component. In addition, our study is the first one to describe secretion of MIF due to a retrovirus infection in humans. Also, we show here that MIF is an up-regulator of HIV-1 replication in vitro, suggesting a novel role for this cytokine in the pathogenesis of HIV-1 infection, and that a continuous MIF release by HIV-1-infected cells can contribute to viral loads and to accelerate progression to AIDS.

First, we investigated if MIF concentrations were elevated in the plasma of individuals infected by HIV-1, and we found that in these patients, levels of MIF were much higher (up to 25 times) than in uninfected individuals. Although there was no positive correlation between plasma viral loads and MIF levels (data not shown), this finding strongly suggests that HIV-1 infection could be responsible for augmenting levels of MIF in vivo. At that point, we could not determine the main source of MIF, whether from HIV-1-infected cells or infected and uninfected cells exposed to HIV-1 antigens (e.g., gp120), or even microbial products translocated from the gut (Brenchley et al., 2006a). However, we could suggest that the amount of circulating virus is not the only factor triggering MIF release.

To assess if HIV-1-infection could be an event capable of inducing MIF release, we infected PBMC with both an R5 or an X4-tropic strain of HIV-1. We observed that both phenotypes elicited MIF secretion in culture, and that infection with an X4-tropic isolate of HIV-1 resulted in a more pronounced MIF secretion than the one mediated by the R5-virus. One explanation for this difference could be the fact that the expression of CXCR4 on cell membranes is more elevated than CCR5, which, in ultimate analysis could result in a higher number of infected cells in vitro and, thus, more cells releasing MIF. Since it has been reported that in nearly 50% of AIDS patients there is a shift in the viral co-receptor usage from R5 variants to X4-tropic HIV-1 isolates (Connor et al., 1997) our findings suggest that patients harboring both phenotypes of HIV-1 variants may have two simultaneous signals for MIF release during the late phases of HIV-1 infection, and that elevated levels of MIF may be detected in infected patients regardless of the harbored viral phenotype.

The augmentation of MIF release secondary to HIV-1 infection, only 7 days after infection might suggest that induction of MIF secretion requires a peak in the viral replication, or even an accumulation of some viral component in the culture. To address this possibility, we treated uninfected cells with HIV-1 gp120 derived from a R5-tropic strain (Ba-L isolate) and observed a significant, although slight, increase in MIF release after 16 h of treatment, showing that an HIV-1 molecule could also induce MIF secretion, although it did not appear to be the main factor inducing elevated amounts of MIF release in infected cultures. Certainly, a replicating virus is a more powerful inducer for MIF release than a soluble glycoprotein, through a mechanism not yet described. Taking in account that gp120 circulates in AIDS patients (Chiodi et al., 1987), this viral protein may contribute to increasing systemic MIF levels as we describe here. Since MIF secretion can be induced by a variety of endogenous/exogenous stimuli (Calandra and Roger, 2003), we understand that it is very difficult to determine the real nature of MIF release in vivo. For an unknown reason, macrophages did not release greater amounts of MIF secondary to HIV-1 infection, despite the fact that macrophages are an important source of MIF (Calandra et al., 1994), suggesting that this phenomenon is probably restricted by cell type.

It has already been shown that MIF is released after microbial stimulation, and LPS from gram-negative bacteria is one of the most potent MIF inducers (Bernhagen et al., 1994; Calandra and Roger, 2003). MIF release secondary to LPS stimulation is dependent on ABC transporters, as shown by the fact that probenecid, an inhibitor of the ABCA1 family of the ABC transporters, blocks MIF secretion upon LPS stimulation (Flieger et al., 2003). The ABCA1 transporter is considered as a major factor responsible for MIF release, and this cytokine is constitutively expressed and stored in intracellular pools (Calandra and Roger, 2003). To investigate if the MIF released by HIV-1 stimulation was also exported from the cells by means of ABCA1 transporters, we treated HIV-1-infected PBMC with probenecid and evaluated MIF contents. We found that treatment with probenecid decreased MIF liberation, indicating that the MIF release upon HIV-1 infection needs the activation of ABCA1 transporters.

The immunoneutralization of MIF in HIV-1-infected cultures revealed the importance of this molecule in viral replication. Addition of anti–MIF antibodies in the cell cultures resulted in diminished viral replication, implying a direct relation between MIF and HIV-1. The association of MIF with HIV-1 was strengthened by the finding that addition of rhMIF to HIV-1-infected cultures led to an augmentation of viral replication by either R5 or X4-tropic isolates. It is important to mention that rhMIF favored HIV-1 replication when this cytokine was added at the same dose (25 ng/mL) observed in HIV-1 plasma patients, suggesting that this MIF concentration could up-regulate HIV-1 production in vivo.

Based on our results shown in Fig. 6, we could suggest that exposure to MIF elicits HIV-1 transcription from LTR, thus favoring viral replication. It has been demonstrated that MIF triggers a signaling pathway that culminates in activation of the three transcription...
factors AP-1, ETS and NF-κB (Amin et al., 2006; Binsky et al., 2007; Calandra and Roger, 2003; Gore et al., 2008; Kleemann et al., 2000; Toh et al., 2006) for which HIV-1 LTR contains binding sites (Copeland, 2005). In this context, cytokines can contribute to HIV-1 production via activation of LTR by positive transcription regulators. For instance, TNF-α is dependent on NF-κB to drive HIV-1 transcription mediated by LTR (Duh et al., 1989; Munoz-Fernandez et al., 1997). In addition, IL-10 cooperates with TNF-α to enhance the binding of AP-1 and NF-κB to the LTR in cells of the monocyte/macrophage lineage (Finnegan et al., 1996). Thus, it is plausible to speculate that HIV-1 LTR transcription stimulated by MIF from HIV-1 LTR encompasses activation and binding of AP-1, ETS and NF-κB to HIV-1 LTR.

Taken together, our results suggest a positive, re-entrant pathway for the establishment of HIV-1 in the host whereby viral infection of cells triggers MIF release, which then augments HIV-1 replication.

Because this study was entirely performed in primary cells (except for data presented in Fig. 6), our results might resemble what is occurring in HIV-1-infected tissues. Thus, MIF may be acting as a detrimental molecule to HIV-1-infected individuals, favoring high viral replication, even supporting viral persistence. Because AIDS patients succumb to opportunistic pathogens and MIF is secreted during the course of a great diversity of infections (Baugh and Bucala, 2002), high levels of MIF induced by co-pathogens could augment HIV-1 replication and contribute to disease progression. Also, it has recently been shown that microbial translocation from the gut to the HIV-1-infected tissues (Brenchley et al., 2006b) and could be occurring in HIV-1-infected individuals, even supporting viral persistence. 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Materials and methods

HIV-1 isolates

Assays of PBMC infection were performed with the monocytotropic, CCR5-dependent isolate HIV-1_Ba-L and with the CXCR4-dependent isolate HIV-1_YTibe, which were donated to us by the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD). Both isolates were expanded in phytohemagglutinin-activated PBMCs, as described elsewhere (Lima et al., 2002).

Reagents and ELISA kits

Recombinant human MIF (rhMIF) was obtained as previously described (Bernhagen et al., 1994); anti-MIF polyclonal antibodies and goat IgG were purchased from R&D Systems (Minneapolis, MN); the ABC transporters inhibitor probenecid was obtained from Sigma-Aldrich (MO, EUA). The recombinant HIV-1 Ba-L gp120 and the p24 antigen. Thus, non-internalized viruses were removed by washing, and macrophages were maintained in DMEM with 10% human serum. Macrophage purity was >90%, as determined by flow cytometry (FACSscan; Becton Dickinson) analysis using anti-CD3 (PharMingen) and anti-CD14 (PharMingen) monoclonal antibodies.

HIV-1 infection

PBMCs were infected with either the HIV-1 R5-isolate Ba-L or the X4-isolate Tybe, using 5 to 10 ng/mL of p24 antigen. After a 2-h incubation, cells were washed to remove excess virus, and culture medium was added back to infected PBMC. HIV-1 replication was evaluated in cell culture supernatants by a commercial ELISA kit, according to manufacturer’s instructions (ZeptoMetrix Co., NY). Macrophages were infected with HIV-1 R5-isolate Ba-L by exposing them overnight to viral suspensions containing 5 to 10 ng/mL of p24 antigen. Thus, non-internalized viruses were removed by washing, and cell monolayers were replenished with fresh medium. HIV-1 production and MIF secretion were measured in cell culture supernatants as described above.

Effect of MIF on HIV-1 replication

To address the role of MIF in HIV-1 replication, HIV-1-infected PBMCs were distributed in 96-well culture plates (2×10^4/well/200 μL) and treated either with rhMIF, anti-MIF antibodies (or isotype control; R&D Systems) immediately after cell infection. Cells were maintained in culture for different time-points, and HIV-1 replication was measured as described above.

Inhibition of ABC transporters

To inhibit the functioning of ABC transporters, PBMCs were treated for 45 min with probenecid (10 μM) prior to HIV-1 infection. After infection, probenecid was added back to cultures, at the same concentration, for the entire period of the experiment (7 days), when cell culture supernatants were collected for quantification of MIF production and HIV-1 replication.
Treatment of PBMC with recombinant HIV-1 gp120

Uninfected PBMCs were treated with recombinant HIV-1 gp120 derived from the R5-tropic HIV-1 Ba isolate (5 μg/mL) for 16 h; then, cell culture supernatants were collected for evaluation of MIF release.

Evaluation of transcription from LTR

CD4+ T Jurkat derivative cell line (1G5) containing stably transfection of luciferase under control of HIV-1 LTR was obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD)/Dr Estuardo Aguilar-Cordova and Dr John Belmont. For evaluation of transcription from HIV-1 LTR, 105 1G5 cells were plated in the absence of stimulus or in the presence of MIF, Tat (100 ng/mL) or PHA (2 μg/mL) for 18 h at 37°C/5% CO2. After this time period, cells were collected, washed twice with PBS and processed according Luciferase Detection Kit (Promega, WI). The luminescence was evaluated on a TD-20/20 luminometer (Turner Designs), following instructions provided by the manufacturer of the kit.

Statistical analysis

All results presented in this study were prepared using Graphpad Prism v4.0 software (San Diego, CA). Statistical analysis calculation was performed using the same software, and the comparisons between values were considered significantly different when the p value was minor than 0.05 using Student’s t test.

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