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RESEARCH ARTICLE

ANTI-INFLAMMATORY EFFECTS OF OZONE IN HUMAN MELANOMA CELLS AND ITS MODULATION OF TUMOUR MICROENVIRONMENT.

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Abstract

Ozone therapy is an effective medical treatment for different diseases like mucositis, psoriasis, acute pain, neurovascular diseases and cancer. Emerging evidence indicates that ozone, a strong oxidant, could effectively improve organ ischemia-reperfusion, herniated disks and skin ulcers in clinical model with interesting anti-inflammatory properties through inhibition of NF-κB activation in acute and chronic disease. The aim of this study is based on the study of the biological effects of ozone in human melanoma cancer cells in order to investigate about its possible use in association to common therapy. Specifically, human melanoma cells were pre exposed or not to pro-inflammatory condition (Lipopolysaccharides) and administration of ozone at different concentration was performed in order to evaluate different biological parameters; cell viability, Measurement of Mitochondrial Matrix Potential (MMP), Evaluation of p65-Nfkb and changes in secretion of interleukins and growth factors involved in melanoma growth, survival and chemo-resistance: IL-1, IL-8, IL-6, TNF-α, IL-9, TGF-β, IL-19, VEGF, MMP-2, MMP-9 and IL-17 by ELISA methods. Results obtained shown ability of ozone to decrease cell viability up to 75% compared to control after 24h of incubation and inhibit all interleukins analyzed and involved in melanoma cell survival and drug resistance. Ozone at 50 µg/ml also decrease of 60% the activation of the pro-inflammatory mediator p65Nfkb and inhibit the Nitric oxide production under pro-inflammatory condition. However, taking the several limitations of this study, further biological preclinical investigations are being carried out in order to understand the potential of ozone as possible adjuvant agent in therapy of melanoma.

Introduction:-

In the last years, the use of ozone as an alternative tool for management of several diseases was sharply increased (Tylicki et al., 2004). Ozone therapy is a nonconventional form of medicine that has been used successfully in the treatment of ischemic disorders like chronic middle ear deafness and tuberculosis are two diseases well treated by using ozone (Quain J,R.,1940 ; Stoker G., 1902). In the field of oncology, the use of ozone was well known only to

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stimulate tissue oxygenation with possible implications in VEGF-oncogens pathway affections (Clavo B et al., 2004) ; in fact, despite being administered over a very short period, ozone therapy improved oxygenation in hypoxic tumors in same clinical cases (Clavo B et al., 2004). From a molecular point of view, ozone has several putative mechanisms of action involving nuclear factor-erythroid 2-related factor 2 (Nrf2), a nuclear transcriptional factor with interesting anticancer and protective actions against neurodegenerative diseases like Alzheimer's and Parkinson's diseases (Sagaiand V et al., 2011). Specifically, the activation of Nrf2 performed by ozone could increase the activity of several tumor suppressor proteins like SOD, catalase (CAT), GSH, GPx, GSH-S-transferase (GSTr), NADPH quinine-oxidoreductase 1 (NQO1), and heat shock protein 70 (HSP70). These effects are substantially comparable to those observed with the use of same nutraceuticals having a great interest in CAM, in particular related to breast cancer chemoprevention (Vecchione R et al., 2016 ; Yucel et al., 2011) . One of the most important factors involved in cancer growth, survival, and resistance to many chemotherapeutics is the anoxic microenvironment; from a clinical point of view, tumor hypoxia is an independent prognostic factor for advanced cancer progression (Nordsmark et al., 2004) in fact patients with hypoxic tumors have significantly lower overall survival or disease-free survival (Simonetti V et al., 2018). One of the most interesting properties of ozone therapy is obviously the impact on the tissue oxygenation; in fact, as recently well demonstrated in a pilot study, ozone therapy could increase oxygenation in several hypoxic tumor tissues and it could be useful as possible adjuvant in chemo-radiotherapy regimens (Clavo B et al., 2004) . Despite the recent clinical studies related to ozone therapy, more biological and biochemical studies are required in order to understand the limitations and the possible adverse effects related to its use in human. We recently published data related to anti-inflammatory effects of ozone in human colon cancer cells and its possible adjuvant effect in combination with conventional chemotherapies like cisplatin and 5-FluoroUracil (Simonetti V et al., 2018). Here we analyzed the multiple effects of different ozone doses on human melanoma microenvironment by studying affections on several interleukins and growth factors under pro-inflammatory conditions understanding its possible and putative role in the complementary management of melanoma.

Materials and Methods:-

Cell Viability:-

The cytotoxicity of ozone was evaluated on the human skin melanoma cell line CHL-1 (purchased from the American Type Culture Collection) looking at their mitochondrial dehydrogenase activity by means of a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method according to the manufacturer's instructions (Dojindo Molecular Technologies Inc., Rockville, MD). The CHL-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc) supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h of appropriate growth, we tested in full medium the effects of ozone (from 10 up to 50 µg/mL) produced with Multiossigen machinery, type Medical 99 IR) following the same procedure reported in literature (Mary VM et al., 2015) and by our group (Simonetti V et al., 2018) by a method that avoided cells from direct expose to ozone. After treatments, melanoma cells were then incubated for 2,6,12 and 24 h under standard conditions. At the end of the incubation period the cells were washed three times with PBS at pH 7.4 and incubated with 100 µl of a MTT solution (0.5 mg/ml in cell culture medium) for 4 h at 37°C. The absorbance readings were acquired at a wavelength of 450 nm with the Tecan Infinite M200 plate reader using I-control software. The relative cell viability (%) was calculated by the formula [A] test/[A] control × 100, where "[A] test" is the absorbance of the test sample and "[A] control" is the absorbance of the control cells incubated solely within culture medium. After evaluating cell cytotoxicity, the total protein content was measured by using the Micro BCA protein assay kit (Pierce). Briefly, the cells were washed with ice-cold PBS and incubated for 15 min in 150 µl cell lysis buffer (0.5% v/v Triton X-100 in PBS), to which 150 µL of Micro BCA protein assay kit reagent (prepared following the instructions of the manufacturer) was added. The absorbance at 562 nm was finally measured on a plate reader. The cytotoxicity measurements were then normalized by the amount of total protein content in each well.

Measurement of Mitochondrial Matrix Potential (MMP):-

MitoProbe™ JC-1 Assay Kit (Thermo Fisher kit) was used to measure the MMP of CHL-1 cells, according to the manufacturer's protocol. Following treatment with Ozone, 1×10^5 cells were cultured in 24-well plates and incubated with 5 µg/ml JC-1 staining solution for 20 min at 37°C. Cells were subsequently rinsed twice with JC-1 staining buffer, and the fluorescence intensities of mitochondrial JC-1 monomers (λ excitation, 514 nm; λemission, 529 nm) and aggregates (λexcitation, 585 nm; λemission, 590 nm) were detected using a monochromator microplate reader. The MMP of CHL-1 cells in each treatment group was calculated as the red/green fluorescence ratio.

NF-κB Transcription Factor Assay:-

Melanoma cells were exposed to ozone at different concentration for 6 h, and then nuclear extracts were analyzed using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA), according to the manufacturer's recommendations. NF-κB complexes were captured by binding to a consensus 5′-GGGACTTTCC-3′ oligonucleotide immobilized on a 96-well plate. Bound NF-κB was quantified by incubating with anti-p65 primary antibody followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and spectrophotometric detection at a wavelength of 450 nm using a microplate spectrofluorometer (xMark Microplate, Spectrofluorometer Biorad, Milan, Italy). Data were expressed as the percentage of NF-κB/DNA binding relative to control cells.

Measurement of nitric oxide:-

In order to verify the effects of ozone on the nitric oxide (NO) release from melanoma cells we have analyzed released nitrite, a stable product of NO in aqueous medium, by using the Griess Reagent System (Promega, Madison, WI, USA) as described in literature (Quagliariello V et al., 2017). Briefly, CHL-1 cells where grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc) supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2, After, $5x10^3$ cells/well were seeded in a 24-well plate and allowed to grow for 24h. Cells were treated with LPS (40 ng/ml) for 6 h or pretreated for 5h with ozone at different concentration. At the end of this time period, culture medium was mixed with an equal volume of sulfanilamide solution (1%v/v in 5% v/v phosphoric acid) and of N-1-naphtylethylenediamine dihydrochloride solution (0.1% v/v in water). The absorbance was measured at 540 nm by using spectrophotometer (xMark Microplate, Spectrofluorometer Biorad, Milan, Italy). Nitrite concentrations were determined from a calibration curve of standard 0.1 M sodium nitrite concentrations 0.5–50 μM against absorbance.

Anti-Inflammatory Tests:-

The expression of IL-1, IL-8, IL-6, TNF-α, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17 by human melanoma cells was evaluated with ELISA, as described in literature (Quagliariello V et al., 2017 ; Quagliariello V et al., 2016). Briefly, CHL-1cells (1.2×10^5 cells/well) were seeded in 12-well plate in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc) supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 After pre-incubation for 24 h and starvation in serum-free medium for 2.5 h, the cells were treated with or without 0.1 ml of a full cell culture medium added with 20, 30, and 50 μg/mL of ozone for 5 h before exposure to LPS (40 ng/ml) for 12 h, in order to stimulate inflammation. After that, culture supernatants were collected, centrifuged to pellet any detached cells, and measured using ELISA methods performed according to the manufacturer's instructions. The sensitivity of these methods were less than 10 (pg/ml), and the assay can accurately detect cytokines in the range of 1–32000 pg/ml.

Statistical Analysis:-

The difference between experimental groups was investigated by a one-way analysis of variance (ANOVA) and by a subsequent Turkey's multiple comparison test in Sigma Plot Software. For statistical analysis of all data, $p < 0.05$ was regarded as the lowest acceptable threshold for significance.

Results:-

Cell Viability

As shown in Figure 1, ozone has a time and concentration dependent cytotoxicity against human melanoma cells, however only after 24h of incubation we observe an IC50 that corresponding to 40 μg/ml (Figure 1, A). The best effects were obtained at 50 μg/ml of ozone with a 75% of cytotoxicity after 24h of incubation. As shown in Figure 1B, there was a clear time dependent cytotoxicity at 30 and 50 μg/ml of ozone.

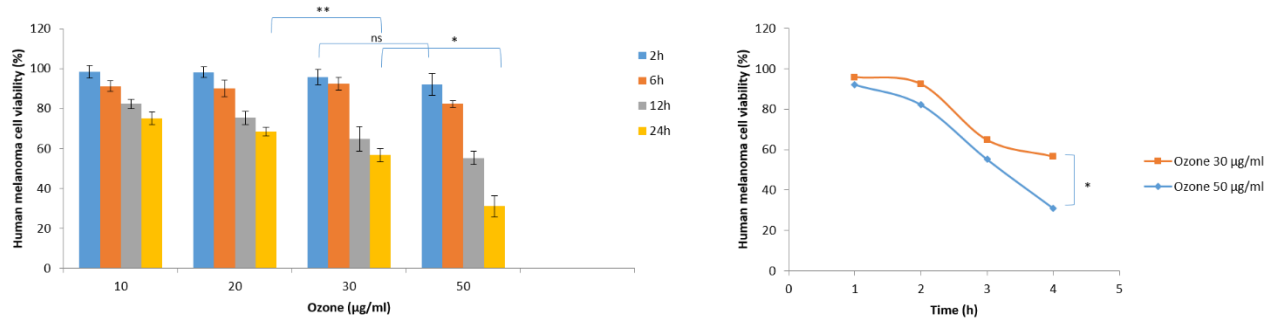

Figure 1:-Melanoma cell viability (± SEM) of CHL-1 cell line performed by modified MTT method as in function of the incubation time and ozone concentration. *p<0.001 ;**p<0.05; ns: non significant.

Measurement of Mitochondrial Matrix Potential (MMP)

To understand the underlying mechanism of inhibited cell proliferation and migration, the MMP were investigated in CHL-1 cells following treatment with ozone. Following treatment with ozone the MMP of CHL-1 cells was significantly decreased of around 45% compared with the control cells (p<0,001). To determine whether ozone affects mitochondrial biogenesis, cells were treated with ozone for 12 h. Mitochondrial ATP synthesis was measured, and the results demonstrated that treatment with ozone significantly decreased ATP content (of around 50%) compared with the untreated cells (p<0,001).

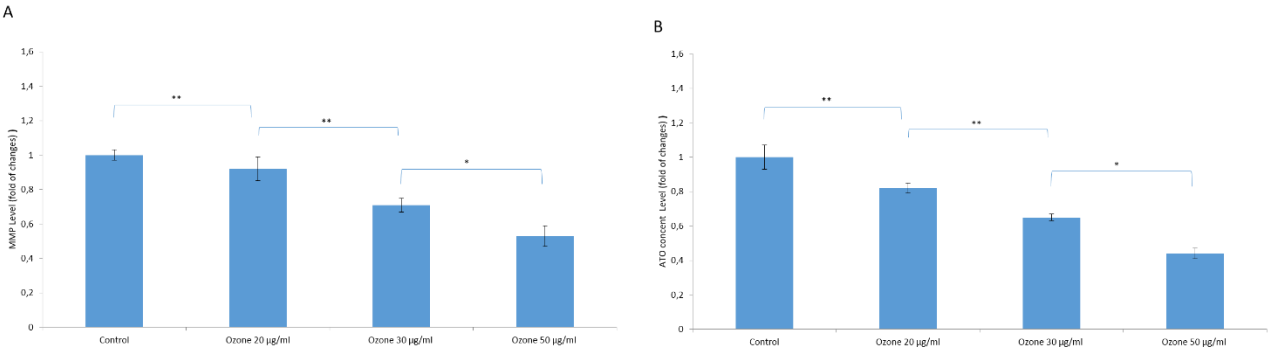

Figure 2:-A: The MMP of the CHL-1 cells determined using a JC-1 kit, relative to the Cont cells. B: Mitochondrial ATP generation decreased following treatment with ozone, relative to the Cont cells. Values are presented as the mean ± standard error of the mean (n=3).

NF-κB Transcription Factor Assay:-

The impact of NF-κB –related pathways on growth and progression of cancer cells has been observed in multiple human carcinomas like melanoma (Lehraiki A et al., 2015. To investigate whether ozone affects NF-κB activation, which is critical for transcriptional activity, the DNA binding activity of NF-κB was analyzed by ELISA. As shown in Figure 3, ozone significantly decreased NF-κB activation in a dose-dependent manner in melanoma cell line also at very low concentration corresponding to 20 µg/ml (P<0.001) indicating strong anti-inflammatory effects. The best results were obtained at 30 µg/ml with a reduction of 49 % of p65-Nfkb binding activity compared to only LPS treated cells (p<0,001). Increasing ozone concentration over 30 µg/ml did not shown more anti-inflammatory activity related to NF-Kb activity indicating a plateau phase.

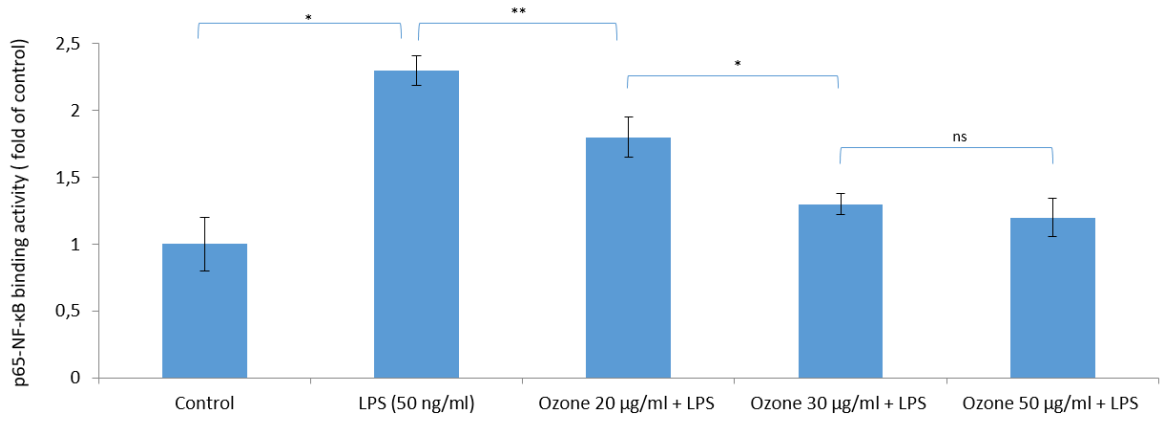

Figure 3:-Effects of Ozone on NF-κB activation. Human melanoma cells were pretreated with various concentrations of Ozone (20, 30 and 50 µg/ml) for 24 h. Nuclear extracts were prepared and NF-κB activation was measured by enzyme-linked immunosorbent assay. Quantified data are expressed as the mean ± standard error of the mean of three experiments. *p<0.001, **p<0.05 ; ns: not significant.

Measurement of nitric oxide

The nitric oxide (NO) produce, by interacting with superoxide anion, peroxynitrite which is implicated in melanoma cell growth and survival (Tanese K et al., 2012). Under pro-inflammatory conditions as well known, melanoma cells can produce NO in fact , as clearly shown in Figure 4, cells significantly increased NO production under pro inflammatory conditions. Treatments with ozone determine significant inhibition of NO production at all tested concentrations; as example. at 30 µg/ml , NO production was inhibited of 49 % with a maximum of 69,5 % of inhibition at 50 µg/ml.

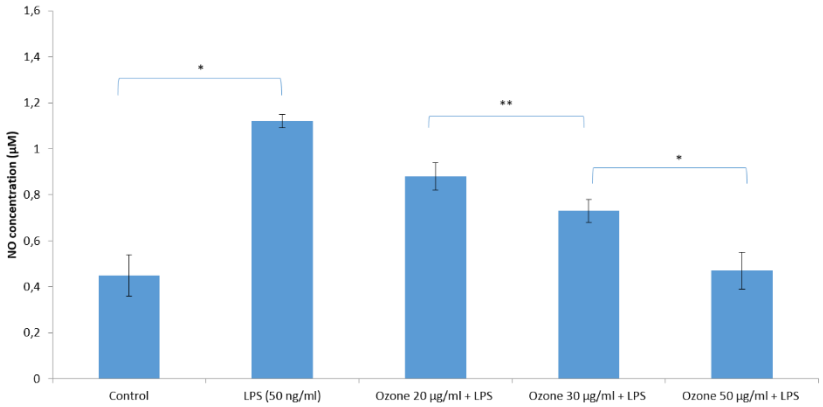

Figure 4:-Measurement of Nitric Oxide Production expressed as NO concentration (µM) in melanoma cells (5000 cells/well). Cells were pretreated with or without Ozone for 5 h prior to stimulation with 40 ng/ml of LPS for 24 h. *p<0,001 ; **p<0,05 ; ns: not significant.

Anti-Inflammatory Tests

Considering the well-established anti-inflammatory activity of ozone we have investigated how it could affects IL-1, IL-8, IL-6, TNF-α, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17 production in melanoma cells under pro-inflammatory conditions incubating cells with LPS at a dose of 40 ng/ml. First of all, incubation of cancer cells only with LPS determine a significant stimulation of all analyzed interleukins and growth factors compared to the untreated one due to their binding with Toll Like Receptor type 4 (TLR4) leading to an up-regulation of interleukins mRNA expression and their secretion (Figure 5). These effects are already demonstrated in other published work (Takazawa Y et al., 2014). However, pretreatment with ozone decreased significantly the level of all molecules analyzed in a concentration dependent manner (Figure 5). Specifically, ozone at 30 µg/ml decrease of 19, 29, 50, 24, 16, 17, 15, 26, 8, 9 and 21% the levels of IL-8, IL-6, TNF-α, IL-1, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17, respectively, compared to only LPS treated cells (p<0,001 for all). By increasing at 50 µg/ml the

concentration of ozone it was obtained a reduction of 33, 37, 64, 36, 35, 33, 34, 40, 29, 25 and 35% of IL-1, IL-8, IL-6, TNF-α, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17 secretion, respectively ($p<0,001$ for all, compared to only LPS treated melanoma cells).

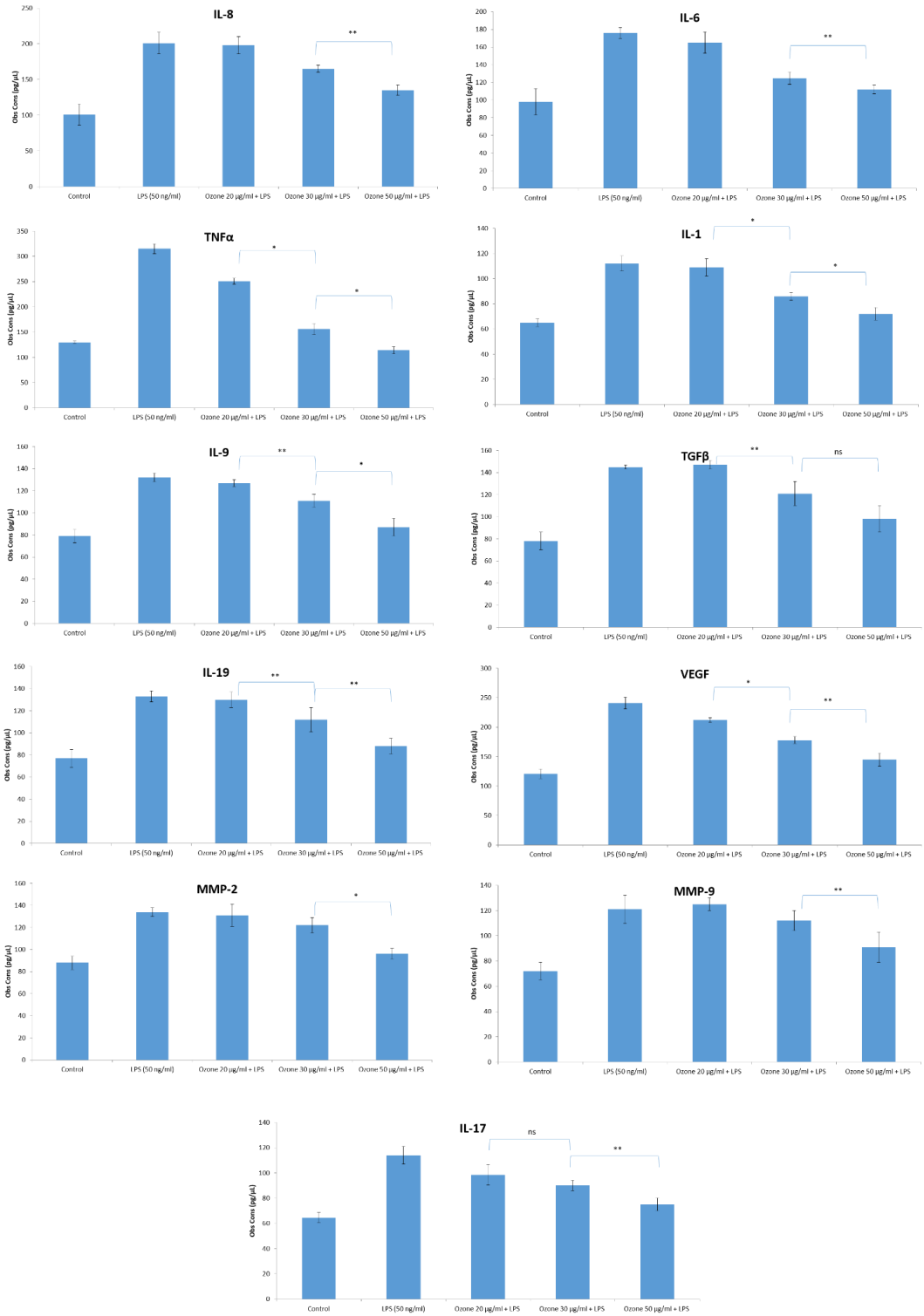

Figure 5:-Anti-inflammatory properties of ozone at different concentration on IL-1, IL-8, IL-6, TNF-α, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17 production by melanoma cells (at a density of 1.2 x 10^5 cells/well). Cells

were treated with or without ozone (from 20 up to 50 µg/ml) for 5h before exposure to LPS (40 ng/ml) for 12 h. *p<0.001, **p<0.05 ; ns: not significant.

Discussion:-

Melanoma microenvironment is one of the most important biochemical factors involved in cancer cell initiation, growth and progression. Melanoma microenvironment is composed by a multitude of interleukins that acts synergistically on their receptors and activate multiple pathways involved in cancer cell metabolism and survival; most of them are based on the activation of pro-inflammatory proteins and growth factors like NfKb and Akt. Nitric oxide has a key role in melanoma immuno resistance and is involved in metastatic processes in several clinical studies. Ozone is an innovative method for decrease inflammation status in preclinical and clinical experiences. Our research group recently demonstrated the abilities of ozone in increase 5 Fluoro-Uracile and Cisplatin cytotoxicity and decrease interleukin secretion in human colon cancer cells (Simonetti V et al., 2018) . Herein it was demonstrated for the first time multiple biological and biochemical properties of ozone in human melanoma cells that start from cancer cell growth inhibition (Figure 1) in anti-inflammatory effects. Of particular interest is the p65 Nfkb inhibition by ozone considering the well known role of this protein complex in the genesis of VEGF, oncogens and several pro-inflammatory interleukins. This anti-inflammatory effect was also previously demonstrated by other groups (Huth KC et al., 2007). In fact, on the basis of this result, we planned and performed quantitative assays of interleukin secretion by melanoma cells under pro-inflammatory condition. In fact, LPS increase IL-1, IL-8, IL-6, TNF-α, IL-9, TGFβ, IL-19, VEGF, MMP-2, MMP-9 and IL-17 secretion of cancer cells confirmed the key role of inflammation in angionenesis, grotwth e pro-metastatic properties in melanoma cells. Interestingly, as shown in Figure 5, Ozone is able to decrease in concentration dependent manner all of these interleukins and growth factors. Of particular interest is the effect on VEGF and Metalloproteases type 9 and 2 secretion considering the well-known predisposition to the metastatic process of melanoma in cancer patients opening interesting clinical insights into the possible chemopreventive management of ozone not only in melanoma but also in other diseases suggesting as possible and investigatable tool in the management of pro-inflammatory cancer microenvironment.

Conclusion:-

The results of the present study demonstrated for the first time that treatment with ozone significantly reduced the MMP of human melanoma cells and decrease pro-inflammatory interleukins secretion affecting cell viability and nitric oxide production. In addition, treatment with ozone significantly decreased melanoma cell mitochondrial bioenergetics, seen as mitochondrial ATP levels and decrease p65-Nfkb production in concentration dependent manner. In conclusion, the results of the present study demonstrate for the first time that treatment with ozone has anti-inflammatory effects on human melanoma cells through the downregulation of mitochondrial function. The present study indicates that ozone is a potentially effective tool for the management of melanoma microenvironment and it could be applied in combination to conventional therapy or surgery, in order to avoid the tumour dormancy phenomenon. Tumour dormancy that does exist in cutaneous melanoma and likely occurs through impaired angiogenesis and immune regulation. Metastatic dormancy of melanoma has not received sufficient attention, most likely because once detectable, metastasis is almost invariably fatal and, understandably, the focus has been on finding ways to prolong life of patients with overt recurrences. Based on these findings, we are planning preclinical studies by using ozone in association with common anticancer drugs as new association therapy of melanoma.

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Conflicts of Interest:-

The authors also declare that Dr. Simonetti is the President of the KAOS ONLUS Association and a board member of SIOOT, and Dr. Franzini is the President of SIOOT.

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