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Original article

Ozone therapy prevents the onset of dysplasia in HPV16-transgenic mice—A pre-clinical efficacy and safety analysis

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ABSTRACT

Infection with high-risk human papillomavirus (HPV), most often HPV16, is associated with the development of anogenital and oropharyngeal cancers. Recently, ozone therapy was reported to have considerable efficacy against rabbit VX2 tumors, induced by the cottontail rabbit papillomavirus. The present study aims to determine whether similar results can be obtained in HPV16-transgenic mice, possibly paving the way for new therapeutic options against HPV-induced cancers.

HPV16-transgenic and wild-type, female, 20 weeks-old mice were injected intraperitoneally with medical O_3/O_2 (80 mL/kg, at O_3 50 µg/mL), once a day, for 5 consecutive days. The animals were sacrificed at 25 weeks-old, and skin samples were analyzed histologically to study tumour progression. Blood and internal organ samples were used to study toxicological parameters.

85.7% of untreated transgenic mice showed dysplastic skin lesions, compared with 28.6% of O_3-treated mice. This was associated with a marked reduction of dermal inflammation associated with those lesions. No significant changes were observed in any toxicological parameters.

These preliminary results support the hypothesis that O_3 therapy is effective against papillomavirus-induced lesions, particularly against those induced by the most common high-risk virus, HPV16. Further studies are needed to confirm the mechanisms underlying these effects.

1. Introduction

Human papillomaviruses (HPV) are a group of epitheliotropic viruses associated with several types of cancer, most prominently cancer of the uterine cervix. Until now, more than 150 HPVs have been fully sequenced, and many more types are expected to be recognized in the future [1]. In addition to cervical cancer, these viruses are also responsible for cancers of the vagina, vulva, penis, anus and oropharynx [2]. Some HPV genotypes, known as “high-risk” types, are more aggressive, causing persistent infections that may originate cancers. Within high-risk genotypes, types 16 and 18 are responsible for approximately 70% of all cervical cancer cases worldwide [3]. On the

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other hand, the genotypes linked with benign disease are rarely found in connection with cancer and are designated as “low-risk” types [4,5]. Vaccination is a highly effective preventive method, but therapeutic vaccination strategies are still in development [1,6]. Therapy for localized cervical cancer is based on surgery, but various radio- and chemotherapy modalities are available for systemic disease [7]. More effective therapeutic approaches are desirable, particularly for systemic disease, and immunotherapies are expected to bring significant improvements to this field [8]. Ozone (O_3) is a strong oxidant which has the capacity to react with most organic and inorganic substances. O_3 reacts with phospholipids on cell membranes to form aldehydes and peroxides, most commonly hydrogen peroxide (H_2O_2), which activates various metabolic routes in erythrocytes, leucocytes and platelets, acting as a mediator of the immune response [9]. Ozone therapy has been already used against acute and chronic bacterial, viral and fungi infections [10]. Recently, ozone therapy was shown to have a tumoricidal effect against the auricular VX2 progressive tumor on rabbits, which was mediated by tumour-infiltrating leukocytes [11]. The authors proposed that O_3 directly or indirectly activated leukocytes to target and destroy tumour cells, although the precise mechanism underlying this activation remains unknown. Although the Shope cottontail rabbit papillomavirus, whose oncogenes drive the VX2 tumour, has long been used as a model to study HPV [12], a closer model featuring a high-risk HPV would be more easily translatable. Accordingly, the goal of this experimental work was to evaluate the effects of O_3 on HPV16-transgenic mice. These animals closely mimic human disease and have already been used by our group for studying the immune response to HPV-induced lesions and its regulation by COX-2 inhibitors [13] and by immunosuppressive agents [14].

2. Materials and methods

2.1. Mice

K14HPV16 mice were donated by Drs. Jeffrey Arbeit and Douglas Hanahan, from the University of California, through the USA National Cancer Institute Mouse Repository. Fourty female 20 weeks-old mice were used, including twenty transgenic ($HPV16^{+/-}$) and twenty wildtype ($HPV16^{-/-}$) animals. Animals were genotyped as previously described [15,16]. This study was approved by the Universidade de Trás-os-Montes e Alto Douro ethics committee (approval no. 10/2013) and the Portuguese Veterinary Directorate (approval no. 0421/000/000/2014).

2.2. Experimental design

The animals were maintained in accordance with the Portuguese (Decreto-Lei 113/2013, dated August the 7th) and European (EU Directive 2010/63/EU) legislation and the ARRIVE guidelines, under controlled conditions of temperature (23 ± 2 °C) light-dark cycle (12 h light/12 h dark) and relative humidity (50 ± 10%). The animals were housed in hard polycarbonate cages coated with corn cob. Water and food were provided *ad libitum*. The animals were checked daily to ensure their well being. The forty mice were divided into 4 groups: group 1 ($HPV16^{-/-}$, n = 10, without treatment); group 2 ($HPV16^{-/-}$, n = 10, O_3/O_2 treatment); group 3 $HPV16^{+/-}$, n = 10, without treatment) and group 4 ($HPV16^{+/-}$, n = 10, O_3/O_2 treatment). The O_3/O_2 mixture (50 μg/mL) was obtained through a medical ozone system (EMS, model OM-200). At 20 weeks-old, treated animals were administered the O_3/O_2 mixture intraperitoneally, once daily, for 5 consecutive days, at 80 mL/kg (adapted from Schulz et al. [17]). The mice's weights, as well as their water and food consumption were recorded weekly. At 25 weeks old, the animals were sacrificed using intraperitoneal ketamine and xylazine followed by cardiac puncture and exsanguination, as recommended by FELASA. Ponderal weight gains were calculated as previously described (Faustino-Rocha et al. [18]). A complete necropsy of the animals was performed, where both cutaneous lesions and internal organs (liver and spleen) were collected for histopathological analysis. The liver and spleen were selected because this mouse strain shows a high incidence of hepatitis and splenitis [19], which could be aggravated by the treatment. Samples for histopathology were fixated in 10% formaldehyde solution.

Table 1
Incidence of skin lesions.

Experimental groups	*n*	Normal skin	Hiperplasia	Displasia
Group 1 (untreated $HPV16^{-/-}$)	10	10/10 (100%)	0/10 (0%)	0/10 (0%)
Group 2 (O_3-treated $HPV16^{-/-}$)	10	10/10 (100%)	0/10 (0%)	0/10 (0%)
Group 3 (untreated $HPV16^{+/-}$)	7	0/7 (0%)	1/7 (14.3%)	6/7 (85.7%)
Group 4 (O_3-treated $HPV16^{+/-}$)	7	0/7 (0%)	5/7 (71.4%)	2/7 (28.6%)

2.3. Hematological and biochemical analyses

A microhematocrit was calculated for each blood sample and plasma was conserved at −80 °C for further biochemical analysis. Capillary tubes were used to calculate microhematocrit values, which were centrifuged at 4500*g* for 5 min. The concentration of total serum proteins, and the activity of the alanine aminotransferase were determined through spectrophotometric methods using an autoanalyzer (Prestige 24i,. Cormay PZ).

2.4. HPV16- associated skin lesions

The formaldehyde-fixed skin samples were included in parafin blocks and stained with hematoxylin and eosin (H&E) for observation under optical microscopy and histological classification, as previously described [20]. The skin samples were classified as normal skin, epidermal hyperplasia and epidermal dysplasia. Total infiltrating leukocytes and specific leukocytic populations (neutrophils, macrophages, lymphocytes, plasma cells, mast cells) were counted in H&E/stained sections, using high-power (×400) fields, on the basis of their typical morphology. Leukocyte counts were expressed as mean values ± standard deviation to the mean.

2.5. Histological organ analysis

Liver and spleen samples were used for histological analysis, based on previous observations showing that this model displays prominent hepatitis and splenitis [19]. Liver samples were classified as normal liver, or hepatitis with 3 grades of severity (grades I–III). The spleen samples were classified as normal spleen, splenic white pulp hyperplasia and granulomatous splenitis.

2.6. Statistical analysis

The data were analyzed using IBM SPSS software, version 17. Results were expressed as mean ± standard error. The ANOVA statistical analysis was performed following a Bonferroni test, to determine whether the differences between groups were statistically significant ($p < 0.05$). A Chi-squared test was performed for the histological lesions, and a Games-Howell test was used to study differences in leukocytic infiltration between groups.

3. Results

3.1. General results

Six animals died during this experiment (three animals from group 3 and three from group 4). Transgenic animals showed reduced final body weights compared with matched wild-type animals, regardless of

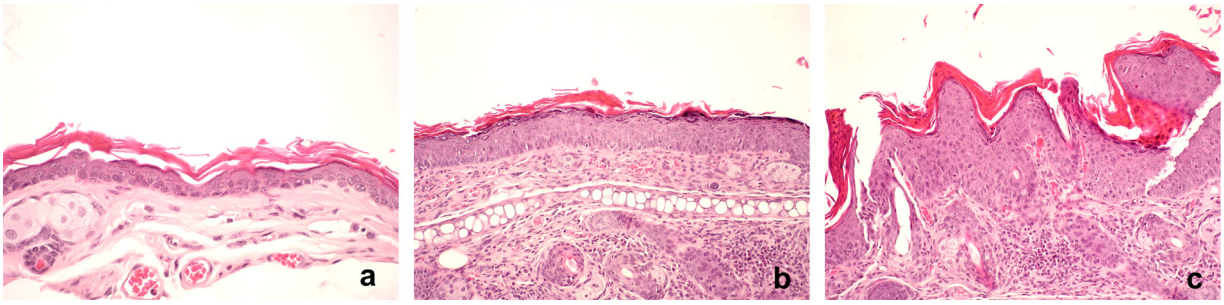

Fig. 1. Histopathological changes induced by HPV16 oncogenes in FVB/n mice, hematoxylin and eosin stain: Normal skin histology, 200× (a); Epidermal hyperplasia, 200× (b); Epidermal dysplasia, 200× (c).

Table 2
Leukocyte counts on skin samples from ozone-treated and untreated wild-type and K14HPV16 mice. Data are the mean ± standard deviation.

Group	Macrophages	Neutrophils	Lymphocytes	Mast cells	Plasma cells	Total leukocytes
1- HPV$^{-/-}$ (n = 12)	0.5[a] ± 0.5	0.1[b] ± 0.3	0.7[a] ± 0.7	0.7[a] ± 0.7	0.1 ± 0.3	2.2[a] ± 1.0
2- HPV$^{-/-}$ O_3 (n = 8)	0.6[a] ± 0.5	0.3[b] ± 0.5	0.8[a] ± 0.2	0.8[a] ± 0.8	0.5 ± 0.7	3.0[a] ± 0.9
3- HPV$^{+/-}$ (n = 12)	4.6 ± 1.9	5.6 ± 2.1	3.9 ± 1.3	5.0 ± 2.4	0.9 ± 0.7	19.9 ± 5.4
4- HPV$^{+/-}$ O_3 (n = 12)	3.6 ± 1.8	1.4[b] ± 1.0	3.4 ± 1.1	3.1 ± 2.4	1.1 ± 1.0	12.7 ± 5.6

[a] Statistically different from group 3 and 4 (p < 0.05).
[b] Statistically different from group 3 (p < 0.01).

Table 3
Kidney, liver, lungs, heart and spleen relative weights (mean ± standard error).

Groups	Left kidney	Right kidney	Liver (mg)	Lungs	Heart	Spleen
Group 1 (HPV16$^{-/-}$)	0.0058 ± 0.0003	0.0054 ± 0.0002	0.0479 ± 0.0003	0.0067 ± 0.0002	0.0045 ± 0.0002	0.0043 ± 0.0002
Group 2 (O_3 HPV16$^{-/-}$)	0.0064 ± 0.0003	0.0063 ± 0.0003	0.0526 ± 0.0025	0.0074 ± 0.0003	0.0051 ± 0.0002	0.0055 ± 0.0003
Group 3 (HPV16$^{+/-}$)	0.0073 ± 0.0003	0.0070 ± 0.0004	0.0749 ± 0.0058	0.0072 ± 0.0003	0.0057 ± 0.0002	0.0104 ± 0.0016
Group 4 (O_3 HPV16$^{+/-}$)	0.0065 ± 0.0001	0.0065 ± 0.0002	0.0647 ± 0.0012	0.0073 ± 0.0003	0.0056 ± 0.0002	0.0083 ± 0.0006

Table 4
Total proteins and alanine aminotransferase (mean ± standard error).

Experimental groups	Total proteins (g/L)	Alanine aminotransferase (U/L)
Group 1 (untreated HPV16$^{-/-}$)	48.68 ± 1.57	36.43 ± 3.10
Group 2 (O_3-treated HPV16$^{-/-}$)	53.86 ± 1.66	38.96 ± 3.11
Group 3 (untreated HPV16$^{+/-}$)	55.46 ± 1.81	45.62 ± 3.16
Group 4 (O_3-treated HPV16$^{+/-}$)	53.93 ± 1.72	43.81 ± 2.98

the ozone treatment and ponderal gain was greater in groups 1 (6.7%) and 2 (8.2%) when compared with groups 3 (3.5%) and 4 (1.2%). Groups 3 and 4 consumed approximately twice as much water compared with groups 1 and 2. The consumption of food was similar in all groups. No statistical differences were observed between treated and untreated animals for any of these parameters.

3.2. Efficacy against HPV16-induced lesions

The histological results concerning skin samples are represented on Table 1. While wild-type animals showed normal skin histology, all transgenic animals showed epidermal hyperplasia or dysplasia. 85.7% of all untreated animals (group 3) showed dysplastic lesions, which was only present in 28.6% of treated mice. The histological images representing the three different situations are compiled on Fig. 1. This difference did not reach statistical significance. Transgenic animals showed higher leukocyte counts compared with their wild-type counterparts. Ozone-treated (group 4) mice showed lower leukocytic infiltration compared with untreated (group 3) mice, particularly concerning the infiltration of neutrophils (p < 0.01). Data concerning leukocytic populations in skin samples from each group are summarized in Table 2.

3.3. Toxicological analyses

The relative weights of internal organs are represented on Table 3. Serum biochemistry parameters are represented in Table 4. Hepatic and

Table 5
Incidence of liver and spleen lesions.

Experimental groups	n	Normal liver	Hepatitis grade I	Hepatitis grade II	Normal spleen	White pulp hiperplasia
Group 1 (untreated HPV16$^{-/-}$)	10	9/10 (90%)	0/10 (0%)	1/10 (10%)	6/10 (60%)	4/10 (40%)
Group 2 (O_3-treated HPV16$^{-/-}$)	10	10/10 (100%)	0/10 (0%)	0/10 (10%)	8/10 (80%)	2/10 (20%)
Group 3 (untreated HPV16$^{+/-}$)	7	1/7 (14.3%)	5/7 (71.4%)	1/7 (14.3%)	1/7 (14.3%)	6/7 (85.7%)
Group 4 (O_3-treated HPV16$^{+/-}$)	7	1/7 (14.3%)	6/7 (85.7%)	0/7 (0%)	3/7 (42.9%)	4/7 (57.1%)

splenic histological changes are summarized in Table 5. No statistical differences were observed between groups for any of these parameters, nor for microhematocrit values (data not shown).

4. Discussion

K14HPV16 mice are a transgenic strain that expresses all the ORFs of the HPV16 early region. These animals present a range of HPV-related lesions, which develop according to the typical HPV-related multistep process of carcinogenesis, along with the animal's age [19]. The mortality observed in the present experiment seems to be due to the strain's inherent frailty, rather than treatment-related, considering that the animals died in equal proportion on both treated and untreated groups. In fact, the treatment with O_3 didn't influence any of the toxicological parameters now analyzed. The growth retardation observed in these transgenic animals has been reported before, along with their increased water intake [19], and neither were aggravated by the treatment. In line with these finding, the relative weights of internal organs didn't present significant changes and the histological analysis of hepatic and splenic tissues revealed typical changes for this strain [19], in both treated and untreated groups. The fact that the alanine aminotransferase (ALT) level is normal in all groups also supports the hypothesis that the treatment wasn't hepatotoxic, since elevated ALT levels are correlated with liver damage and hepatocellular necrosis [21]. Serum protein levels were also very similar between groups, and do not suggest major changes in albumin and globulin levels, thereby indicating normal hepatic function [22]. The microhematocrit didn't present significant changes either. Taken together, these data suggest that the O_3 treatment was safe in our model, particularly concerning the specific hepatic and splenic frailties of our mouse strain.

The histological analysis of skin lesions indicates that the O_3 treatment prevented the progression of the HPV-associated lesions from the hyperplastic to the dysplastic stage. This effect did not reach statistical significance, probably because of the relatively small sample size. This is in agreement with the findings from another group, which reported that intraperitoneal O_3 induced regression of VX2 auricular tumors in rabbits [11]. Taken together, both studies suggest that O_3 treatments may be effective against lesions induced by papillomaviruses. In particular, our findings suggest that O_3 therapies may be useful against lesions induced by high-risk HPV such as HPV16, which are responsible for the vast majority of HPV-related cancers. Rossmann et al. [11] also demonstrated that the regression of VX2 tumors in rabbits was related to the increased infiltration of intratumoral leukocytes, particularly $CD3^+$ lymphocytes. In K14HPV16 mice, a wide array of leukocytes has been shown to influence carcinogenesis, closely mimicking the tumor microenvironment observed in patients with HPV-related cancers [12]. Mast cells and B lymphocytes appear to promote tumor-associated inflammation and tumor progression [23,24]. $CD4^+$ lymphocytes were also related to immune tolerance and tumor progression [25]. On the other hand, $CD8^+$ T cells are associated with tumor regression, particularly when activated to degranulate and exert their cytotoxic effects over cancer cells, an effect which may be enhanced pharmacologically [13] or blocked by immune-suppressive toxins [14]. In the present study, O_3-treated animals showed reduced dermal leukocytic infiltration compared with untreated animals, mainly reflecting less severe epidermal lesions, and supporting the idea that ozone therapy acts through immunomodulatory mechanisms. Additional studies are under way, using larger experimental groups, in order to confirm this therapy's efficacy, as well as to thoroughly characterize the cellular populations involved, and their kinetics within lesions.

5. Conclusion

This experiment suggests that the O_3 therapy tested didn't induce adverse effects in this mouse strain and for the present experimental conditions. Importantly, the therapy partially prevented the progression of hyperplastic to dysplastic lesions, suggesting it may be effective against HPV-induced lesions. Further studies are required, in order to confirm the immunologic mechanisms involved in this process, as well as to test the ability of O_3 therapy to induce the regression (rather than blocking the progression) of HPV-induced lesions.

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