Tumorigenic response in lung tumor susceptible A/J mice after sub-chronic exposure to calcium chromate or iron (III) oxide

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Abstract

Iron oxides are Group 3 (*not classifiable as to its carcinogenicity to humans)* according to the International Agency for Research on Cancer (IARC). Occupational exposures during iron and steel founding and hematite underground mining as well as other iron predominant exposures such as welding are Group 1 (*carcinogenic to humans*). The objective of this study was to investigate the potential of iron as iron (III) oxide (Fe_2O_3) to initiate lung tumors in A/J mice, a lung tumor susceptible strain. Male A/J mice were exposed by oropharyngeal aspiration to suspensions of Fe_2O_3 (1 mg) or calcium chromate ($CaCrO_4$; 100 μg; positive control) for 26 weeks (once per week). Shams were exposed to 50 μL phosphate buffered saline (PBS; vehicle). Mice were euthanized 70 weeks after the first exposure and lung nodules were enumerated. Both $CaCrO_4$ and Fe_2O_3 significantly increased gross-observed lung tumor multiplicity in A/J mice (9.63 ± 0.55 and 3.35 ± 0.30, respectively) compared to sham (2.31 ± 0.19). Histopathological analysis showed that bronchiolo-alveolar adenomas (BAA) and carcinomas (BAC) were the primary lung tumor types in all groups and were increased in the exposed groups compared to sham. BAC were significantly increased (146 %) in the $CaCrO_4$ group and neared significance in the Fe_2O_3 group (100 % increase; $p = 0.085$). BAA and other histopathological indices of toxicity followed the same pattern with exposed groups increased compared to sham control. In conclusion, evidence from this study, in combination with our previous studies, demonstrate that exposure to iron alone may be a potential risk factor for lung carcinogenesis.

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1. Introduction

Iron oxides are not classified as a human carcinogen (currently Group 3 or *not classifiable as to its carcinogenicity to humans*) according to the International Agency for Research on Cancer (IARC) (IARC, 1987). Occupational exposures during iron and steel founding and hematite underground mining are Group 1 (*carcinogenic to humans*), however (IARC, 2012a,b). In 2017, welding fumes, an exposure in which the predominant metal is iron, were reclassified as Group 1. Interestingly, epidemiological data and animal evidence suggest that welding fumes that do not contain carcinogenic metals [e.g., chromium (Cr) or nickel (Ni)] are attributable to an increased risk of lung cancer (IARC, 2018; Falcone et al., 2018a). These fumes, generated from welding on mild steel, are mostly iron and manganese with approximately 85 % iron. In fact, over a decade ago the IARC acknowledged that there was "an as-yet unexplained common reason" for the increased risk of lung cancer observed in epidemiological studies with both mild steel and stainless steel welding exposures, and it has been suggested that iron may have a causative role (IARC, 2009, 2014; Siew et al., 2008). Iron continues to be highlighted as a potential focus area for cancer causation (Zhang and Zhang, 2015; Torti and Torti, 2013). The effects of iron, specifically in humans, are difficult to assess as the exposure is nearly always mixed with other metals or potential carcinogens (IARC, 2012a,b; Wild et al., 2009; Siew et al., 2008). Of the studies reviewed that specifically relate to iron oxide exposure in humans, the evidence to date suggest bulk iron oxide is not a carcinogen (Pease et al., 2016).

Previously, we reported that iron (III) oxide (Fe_2O_3) enhanced lung tumorigenesis in a two-stage (initiation-promotion) model in lung tumor susceptible A/J mice (Falcone et al., 2018b). The findings complemented the human, and limited animal, evidence that showed that carcinogenic metal-containing and non-carcinogenic metal-containing welding fumes were associated with an increased lung cancer risk (Falcone et al., 2017, 2018a; Kendzia et al., 2013; Matrat et al., 2016; 't Mannetje et al., 2012). Therefore, as a continuation of our studies, examination of iron as an initiator will help to determine its carcinogenic potency. This study investigated the lung tumorigenic response to an unclassified metal (e.g. Fe_2O_3) compared to a known carcinogenic metal [e.g., [Cr(VI)] as calcium chromate or $CaCrO_4$]. Cr(VI) is a common occupational and environmental agent and can present as a mixed exposure with iron in exposures such as welding (IARC, 2012a,b).

2. Materials and methods

2.1. Animals

Lung tumor susceptible male A/J mice, age 5–7 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in an AAALAC-accredited, specific pathogen-free, environmentally controlled facility. Mice were housed two per cage in ventilated cages and provided HEPA-filtered air under a controlled light cycle (12 h light/12 h dark). Animals

were acclimated to the animal facility for 1 week and allowed access to a conventional diet (6% Irradiated NIH-31 Diet, Harlan Teklad, Madison, WI) and filtered tap water ad libitum. All animal studies were approved by the Centers for Disease Control-Morgantown Institutional Animal Care and Use Committee and applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

2.2. Complete carcinogenesis bioassay

Mice were weight- and age-matched and organized into three groups (n = 80/group; sham, Fe_2O_3, and $CaCrO_4$). Mice were exposed by oropharyngeal aspiration, as previously described (Falcone et al., 2018b; Rao et al., 2003), for a period of 26 weeks at a frequency of one time per week (Fig. 1, panel A). Two hundred six mice remained after the exposure period. Body weights were recorded weekly throughout the entire 70 week experimental protocol.

Doses for the $CaCrO_4$ and Fe_2O_3 were 100 μg (cumulative 2.6 mg) and 1 mg (cumulative 26 mg) per exposure, respectively. The relevancy of the Fe_2O_3 dose was approximated using previous estimates for mouse studies of welding fumes (Erdely et al., 2011). Using 5 mg/m^3, the NIOSH and American Conference of Governmental Industrial Hygienists (ACGIH) exposure limits for iron oxide, a ventilation rate of 20 L/min, exposure for 8 h/day and an alveolar deposition of 16 %, the daily estimated alveolar deposition in a human would be 7.7 mg (Raabe et al., 1988). Using surface area (102 m^2 for a human and 0.05 m^2 for a mouse) the equivalent in a mouse would be 3.77 μg/day. The mouse was given 26 mg over 26 weeks representing 27.6 years of human exposure working 8 h/day at the exposure limit of 5 mg/m^3. Therefore, the exposure dose is roughly equivalent to a working lifetime. The positive control of $CaCrO_4$ was 1/10 the dose of Fe_2O_3 or 2.6 mg cumulative dose. This was expected to provide a positive result based on previous Cr studies (Beaver et al., 2009; Steinhoff et al., 1986; Zeidler-Erdely et al., 2008), consistent with the fact the exposure limit of hexavalent Cr [Cr(VI)] is 1/1000, or 5 μg/m^3. The metal oxide suspensions were prepared in 50 μL USP-grade calcium and magnesium-free phosphate buffered saline (PBS) and sham animals were exposed to 50 μL of the vehicle using the same exposure regime. $CaCrO_4$ (product number CDS001277; 156.07 g/mol) and Fe_2O_3 (product number 310050; 159.69 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO). Scanning electron microscopy images of the particles are shown in Fig. 1, panel B and C. Additional characterization including specific surface area, hydrodynamic diameter, and zeta potential was done previously (Falcone et al., 2018b).

Animals were sacrificed at 70 weeks after the first exposure by an overdose of sodium pentobarbital euthanasia solution (Fatal Plus; 100–300 mg/kg intraperitoneal; 390 mg/mL; Henry Schein; Dublin, Ohio) then weighed. Once unresponsive to a toe pinch, mice were euthanized by exsanguination and the abdomen and thoracic cavity were opened and examined for any abnormalities. Grossly observed lung tumors were counted at sacrifice as described previously (Falcone et al., 2017). Gross lung images were taken using an Olympus DP21 digital camera (Olympus America; San Jose, CA). For histopathology analysis, formalin fixed whole lung tissue were embedded in paraffin then a 5 μm standardized section was cut. Slides were stained with hematoxylin and eosin and interpreted by a

contracted board-certified veterinary pathologist. Histopathologic lesions were classified using standard published INHAND terminology (Renne et al., 2009). Neoplastic findings were recorded as present and the number of tumors/section were recorded and non-neoplastic histopathologic findings were graded and recorded using the grading scale derived from Mann et al. (2012). Non-neoplastic findings were scored on the following severity scale: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Foreign material and mineralization were only recorded as either present or not present.

2.3. Statistical analysis

Animals that survived to 70 weeks and were without tumors elsewhere besides the lung were included in the final analyses. Statistical analyses were done using JMP version 12.4 and SAS version 9.4 for Windows (SAS Institute; Cary NC). Histopathological findings using the graded scale were analyzed using nonparametric Kruskal Wallis tests followed by Wilcoxon Rank Sum tests for pair-wise comparisons. Lung tumor incidence was analyzed using a Chi-square test in SAS 'Proc Freq,' utilizing Fishers Exact Test, while tumor multiplicity was analyzed using Poisson regression in SAS 'Proc Genmod'. In cases where overdispersion existed, a negative binomial regression was performed. Body weight measures were analyzed using repeated measures analysis of variance with time as the repeated factor. Additionally, body weight data were evaluated using analysis of covariance with time as the covariate to compare the slopes of the growth curves. For all analyses, a p-value of < 0.05 was set as the criterion for significance.

3. Results and discussion

3.1. Morbidity and mortality

During the post-exposure period 13 sham, 13 $CaCrO_4$, and 8 Fe_2O_3 mice were either euthanized because of ongoing morbidities or found dead. Morbidities included abscesses, pelvic/abdominal/spinal masses, and gastrointestinal bleeds. By 70 weeks, 172 mice remained. The final dataset for the gross tumor counts and body weight analysis includes 156 animals. Eleven mice were removed due to tumors found elsewhere besides the lung and five sham mice were removed because of inadvertent dosing with $CaCrO_4$. Body weight analysis of the slopes of the growth curves revealed that the growth rate increase over time was significantly lower in the $CaCrO_4$ and Fe_2O_3 groups compared to sham (Fig. 2).

3.2. Gross lung tumor analysis

Gross images of $CaCrO_4$- and Fe_2O_3-exposed (panel A and B, respectively) mouse lungs 24 h post-fixation are shown in Fig. 3. The black arrows indicate lung tumors which were opaque and had white coloration. Fe_2O_3-exposed lungs were consistently red in color with black deposits throughout while particle deposition was only occasionally visible upon gross exam in the $CaCrO_4$-exposed lungs. At 70 weeks, oropharyngeal aspiration exposure to $CaCrO_4$ or Fe_2O_3 significantly increased gross-observed lung tumor multiplicity (average tumor number/mouse lung ± SE) compared to sham (Table 1). Multiplicity in the $CaCrO_4$ and Fe_2O_3 groups were 9.63 ± 0.55 and 3.35 ± 0.30, respectively, compared to 2.31 ± 0.19 in the sham animals. As expected, gross tumor incidence was not different among the groups and was 90, 94, and 100 % in the Fe_2O_3, sham, and $CaCrO_4$ groups, respectively, at 77–79

weeks of age. Grossly observed background tumor frequency in the A/J mouse, as reported in the literature, can range from 31 to 40% between 43–53 weeks of age and continue to increase to near 100 % by approximately 2 years of age (Curtin et al., 2004; Groch et al., 1997; Witschi et al., 2004). The spontaneous tumor rate in this model is a known limitation and a less reliable indicator of carcinogenicity compared to lung multiplicity. Therefore, multiplicity remains the primary indicator of a positive tumorigenic response in the A/J mouse (Shimkin and Stoner, 1975; Witschi, 2005).

3.3. Histopathological analysis

It was shown by histopathological evaluation (Table 2) that the predominant neoplastic lesions observed in the lungs were bronchiolo-alveolar adenomas and carcinomas (BAA and BAC, respectively). These tumor types were the most prevalent in the $CaCrO_4$ group (30 tumors in 47 mice), followed by the Fe_2O_3 (21 tumors in 46 mice), and least prevalent in the sham group (11 tumors in 38 mice). In the shams, BAA were generally 3–5 mm in diameter, well-circumscribed, round, expansile, and densely cellular neoplasms comprised of tubules and papillary structures lined by uniform cuboidal neoplastic epithelial cells. BAC were typically > 5 mm in diameter, well- to poorly-circumscribed, irregularly shaped, expansile, and densely cellular neoplasms composed of larger, pleomorphic, more mitotically active neoplastic epithelial cells arranged in tubules, trabeculae, papillary structures, and/or less defined, solid areas. In both exposed groups, BAA were similar to those described in the sham animals. BAC were also similar to those of the sham group, but were sometimes larger (15–20 mm diameter) with areas of necrosis and scirrhous reaction (Fig. 3, panels C&D).

Tumor multiplicity and incidence was statistically significant for BAC, as well as BAA, in the $CaCrO_4$ group when analyzed individually and in combination. While gross-observed tumor multiplicity reached statistical significance in the Fe_2O_3 group, the histopathological analysis for BAC and BAA + BAC did not. Fe_2O_3 caused a 59 % increase in BAA + BAC and a doubling of BAC ($p = 0.085$), however, which indicates a trend toward an increase in the malignant tumor type in this group compared to sham. It should be noted that the sample size evaluated microscopically was less than the gross-observed because selected lungs were flash-frozen for later analysis at sacrifice. This likely decreased the overall statistical power to detect a significant difference for the histopathological evaluation of the Fe_2O_3 group. Incidences of cystic keratinizing epithelioma, nonkeratinizing epithelioma (2 $CaCrO_4$ mice) and lymphoma (1 sham mouse) were isolated and not significantly different (data not shown).

Similar to tumor multiplicity and incidence, inflammation and injury in the lung followed the same pattern with the $CaCrO_4$ group the most severe followed by Fe_2O_3 (Table 3). No associated lung injury with PBS exposure was found in the sham group. In the $CaCrO_4$- and Fe_2O_3-exposed groups, the inflammation was significantly increased compared to the sham group and was typically associated with the hyperplastic or neoplastic lesions, but was also associated with foreign material, particularly in the Fe_2O_3 group. In the $CaCrO_4$ group, inflammation was chronic-active and ranged from minimal to marked in severity and was characterized by numerous macrophages, viable and degenerate neutrophils, multinucleated giant cells, and fewer lymphocytes and plasma cells within alveoli and

occasionally bronchioles. Chronic-active inflammation was occasionally associated with basophilic spicules or globules of foreign material (e.g., $CaCrO_4$) that was found in 17 % of the lungs. In the Fe_2O_3 group, chronic alveolar inflammation ranged from minimal to moderate in severity and was characterized predominantly by heavily black pigment-laden macrophages (e.g. Fe_2O_3) with fewer numbers of neutrophils, lymphocytes, and plasma cells. Fe_2O_3, reported as foreign material characterized by abundant extracellular and intracellular black granular pigment, was observed in 46 of 46 lungs (100 %) at 70 weeks. In comparison, the chronic alveolar inflammation in the sham group, likely due to the spontaneous lung tumors, was minimal in severity and characterized predominantly by macrophages with variably abundant eosinophilic cytoplasm.

Type II pneumocyte hyperplasia was another significant non-neoplastic finding in the $CaCrO_4$ group (Table 3). This hyperplasia was peribronchiolar to subpleural in distribution, ranged from minimal to marked in severity, and was characterized by a single layer of cuboidal epithelial cells (type II pneumocytes) lining the alveolar septae. Other predominant findings were bronchiectasis and mineralization. Bronchiectasis ranged from minimal to moderate in severity and was characterized by dilation of the bronchial/iolar lumens occasionally associated with intraluminal mucopurulent exudate. Mineralization, indicative of repeated tissue damage, which was characterized by mineral within bronchial/iolar or alveolar walls and/or associated with chronic-active inflammation.

In the Fe_2O_3 group, significant non-neoplastic findings included hyperplasia of the mucosa-associated lymphoid tissue (MALT) and atelectasis (Table 3). Increased MALT hyperplasia, which ranged from minimal to moderate in severity, was likely a reactive lymphoid response to the exposure. The atelectasis was likely associated with increased inflammation, damage to the pulmonary parenchyma and interference with gas exchange leading to alveolar collapse. Overall, the increased bronchiectasis, mineralization, and atelectasis were presumably reactive responses to $CaCrO_4$ and/or Fe_2O_3 exposure and/or increased numbers of lung tumors.

4. General conclusions

Iron oxides are classified as Group 3 because studies examining whether iron alone was a carcinogen were mostly subject to confounding exposures such as metals or other carcinogens. However, iron industries are classified as Group 1, suggesting iron-rich occupational exposures have the potential to cause lung carcinogenicity. Numerous worker studies have been unable to associate an elevated risk of lung cancer only with carcinogenic metal-containing (e.g., Cr and Ni) stainless steel compared to iron-abundant mild steel welding fumes. Therefore, the IARC classifies welding fumes as a Group 1 because this increased risk was observed regardless of the welding process/method or material/consumable used (IARC, 2018; Langard, 1994; Moulin, 1997; Sorensen et al., 2007). It has been proposed that iron was causative (IARC, 2009, 2014; Siew et al., 2008) and identified it as an area in need of future research, as reviewed in Zeidler-Erdely et al. (2019). Overall, our *in vivo* data supported this notion because it was found that mild steel and stainless steel welding fumes exhibited a similar potency to enhance lung tumorigenesis and Fe_2O_3 alone acted in the same manner (Falcone et al., 2017, 2018a,b). In this study, Fe_2O_3 exposure

significantly increased gross lung tumor multiplicity with a 100 % increase in BAC. The results indicate that iron oxide exposure alone may confer carcinogenicity at or near the threshold of significance and that subsequent, even sub-threshold, exposures could result in a carcinogenic effect. This was supported by our initiation-promotion model, where Fe_2O_3, but not Cr(VI), enhanced lung tumors at levels consistent with that found in welding fume.

Acknowledgements

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A.

Oropharyngeal Aspiration (1x/week):
Sham (PBS 50 μl); CaCrO$_4$ (100 μg); Fe$_2$O$_3$ (1 mg)

Body Weights (weekly)

Gross tumor counts
Histopathology

1   26   70
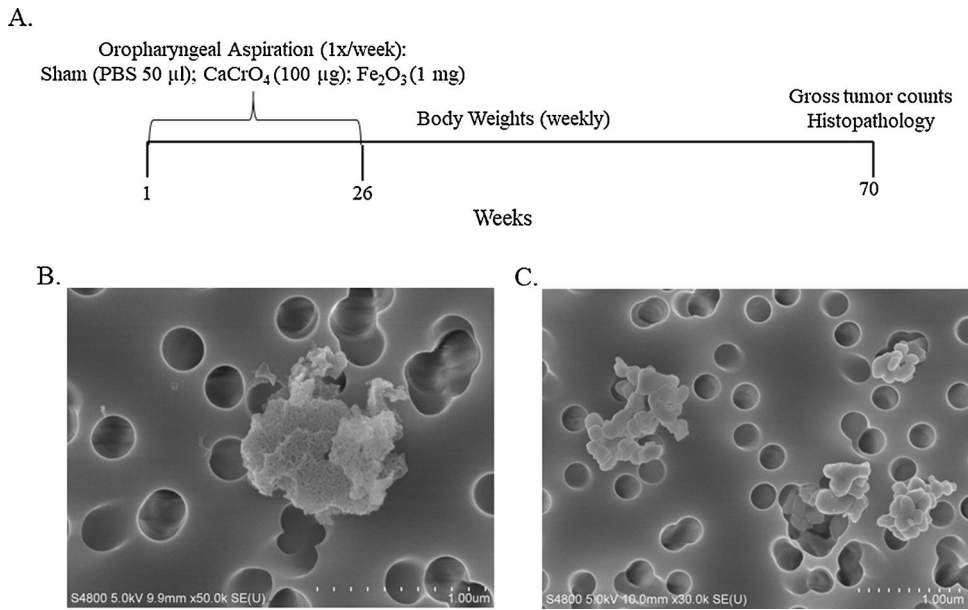
Weeks

B.

C.

Fig. 1.
Experimental protocol (Panel A) for the lung carcinogenesis model to assess CaCrO$_4$ and Fe$_2$O$_3$ as tumor initiators in A/J mice. Mice were exposed to CaCrO$_4$ (100 μg), Fe$_2$O$_3$ (1 mg), or PBS (vehicle; sham; 50 μl) by oropharyngeal aspiration once a week for 26 weeks. Body weights were recorded at week 0, then at each weekly aspiration exposure and weekly thereafter. Mice were sacrificed 70 weeks after the first exposure. Scanning electron images of CaCrO$_4$ (Panel B) and Fe$_2$O$_3$ (Panel C).
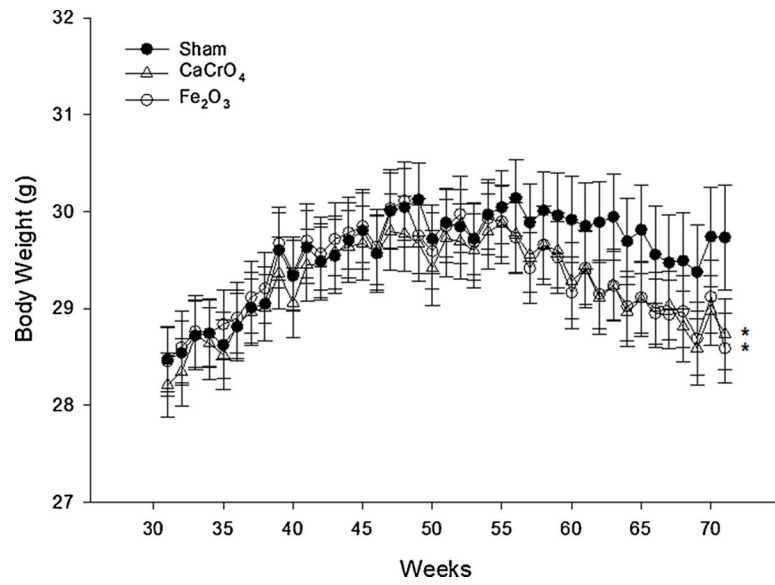
Fig. 2.
Effects on body weight after exposure to $CaCrO_4$ or Fe_2O_3 for 26 weeks. Body weight curves beginning at 30 weeks are shown for surviving animals of the 70-week experimental protocol. The growth rate increase over time was significantly lower in the $CaCrO_4$ and Fe_2O_3 groups compared to sham. Curves were analyzed for the entire 70-week protocol. *p < 0.05-compared to sham.
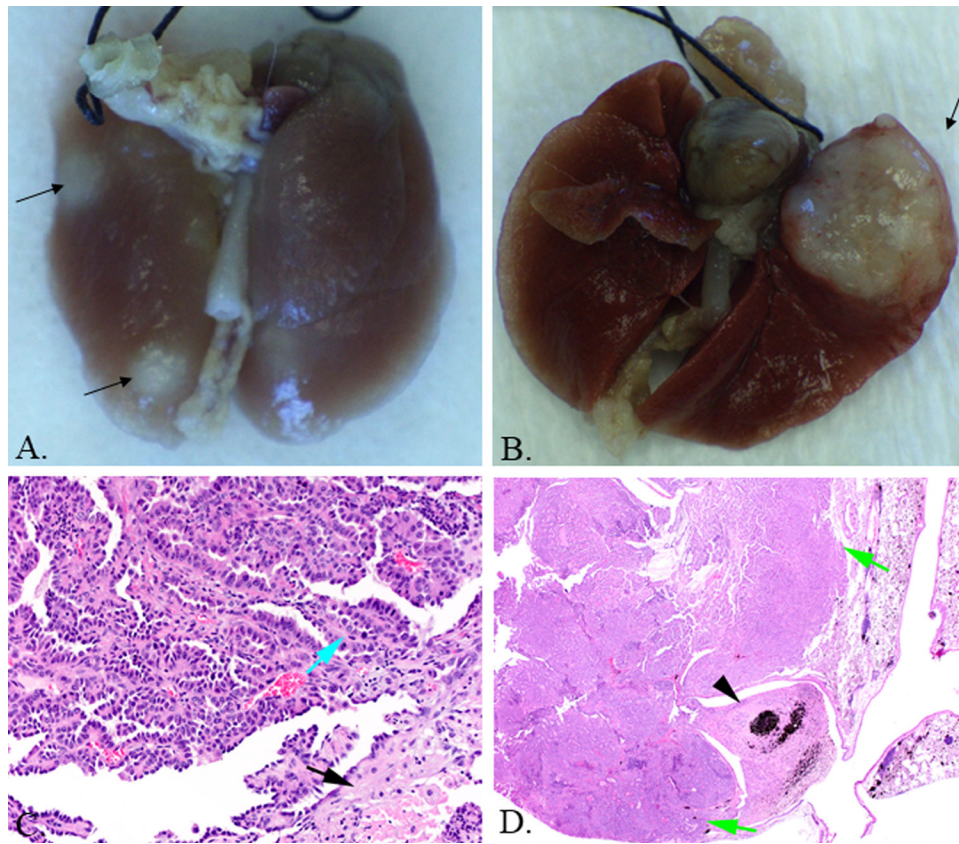
Fig. 3.
Gross images of lung tumors initiated by $CaCrO_4$ or Fe_2O_3 at 70 weeks. Panels A and B represent the lung tumor morphology 24 h after fixation. The arrows (↑) indicate lung tumors.

Photomicrograph images of a BAC (Panel C, 20x magnification) in a $CaCrO_4$–exposed mouse. The BAC was comprised of large, pleomorphic cells forming papillary structures (blue ↑). Scirrhous response (black ↑) was also present. Shown in Panel D (2x magnification) is a BAC in an Fe_2O_3 -exposed mouse. Note the large, expansile, multinodular mass (green ↑) infiltrating into the terminal bronchiole (black arrowhead).

Table 1

Gross-observed total and mean ± standard error of the mean () tumor number across individual lung lobes in A/J mice after sub-chronic oropharyngeal aspiration exposure to $CaCrO_4$ or Fe_2O_3 at 70 weeks.

Exposure	n	Right Apical	Azygos	Cardiac	Diaphragmatic	Left	Total (multiplicity)[*]	Tumor Incidence[*]
Sham	48	14 (0.29 ± 0.11)	14 (0.29 ± 0.08)	13 (0.27 ± 0.06)	27 (0.56 ± 0.12)	43 (0.90 ± 0.13)	111 (2.31 ± 0.19)	94 %
$CaCrO_4$	56	68 (1.21 ± 0.16)[#]	73 (1.30 ± 0.12)[#]	87 (1.55 ± 0.17)[#]	139 (2.48 ± 0.20)[#]	172 (3.07 ± 0.25)[#]	539 (9.63 ± 0.55)[#]	100 %
Fe_2O_3	52	20 (0.38 ± 0.10)	30 (0.58 ± 0.10)[**]	36 (0.69 ± 0.13)[**]	42 (0.81 ± 0.12)	46 (0.88 ± 0.14)	174 (3.35 ± 0.30)[#]	90 %

Abbreviations: $CaCrO_4$ – calcium chromate; Fe_2O_3 – iron (III) oxide.

[*]
Lung tumor incidence was recorded as the percent of tumor-bearing mice out of the total. Lung tumor multiplicity was determined as the average tumor number per mouse lung including mice with no tumors.

[**]
$p < 0.04$ – compared to sham.

[#]
$p < 0.003$ – compared to sham.

Table 2

Histopathologic evaluation of neoplastic lung lesions in A/J mice at 70 weeks after sub-chronic oropharyngeal aspiration exposure to $CaCrO_4$ or Fe_2O_3.

Exposure	n	Tumor multiplicity for BAA and BAC[*]	Tumor multiplicity for BAC	Tumor Incidence[*]
Sham	38	0.29 ± 0.09 (11)	0.13 ± 0.07	24%
$CaCrO_4$	47	0.77 ± 0.13 (36)[**]	0.32 ± 0.08[#]	57 %[**]
Fe_2O_3	46	0.46 ± 0.11 (21)	0.26 ± 0.08	35%

Abbreviations: $CaCrO_4$ – calcium chromate; Fe_2O_3 – iron (III) oxide; BAA - Bronchiolo-Alveolar Adenoma; BAC - Bronchiolo-Alveolar Carcinoma.

[*] Lung tumor multiplicity was determined as the mean ± standard error of the mean tumor number per mouse lung including mice with no tumors, () indicate total numbers of BAA and BAC lesions. Lung tumor incidence was recorded as the percent of tumor-bearing, BAA and BAC combined, mice out of the total.

[**] $p < 0.01$ – compared to sham.

[#] $p < 0.03$ compared to sham.

Table 3

Histopathologic evaluation of non-neoplastic lung lesions in A/J mice at 70 weeks after sub-chronic oropharyngeal aspiration exposure to $CaCrO_4$ or Fe_2O_3.

Exposure	n	Foreign Material*	MALT; Hyperplasia	Atelectasis	Pneumocyte type II; Hyperplasia	Alveolus; Inflammation; chronic	Alveolus; Inflammation; chronic-active	Mineralization	Bronchiectasis
Sham	38	0%	0.18 ± 0.07	0.00 ± 0.00	0.21 ± 0.08	0.73 ± 0.14	0.00 ± 0.00	0%	0.00 ± 0.00
$CaCrO_4$	47	17 %**	0.28 ± 0.08	0.13 ± 0.07	1.36 ± 0.61**	0.00 ± 0.00	1.96 ± 0.66**	74 %**	1.66 ± 0.15**
Fe_2O_3	46	100 %**	1.02 ± 0.10**	0.18 ± 0.07*	0.15 ± 0.05	2.04 ± 0.08**	0.00 ± 0.00	0%	0.07 ± 0.04

Abbreviations: $CaCrO_4$ – calcium chromate; Fe_2O_3 – iron (III) oxide; MALT - mucosa-associated lymphoid tissue.

*
Foreign material (presumptive test article; e.g., $CaCrO_4$ or Fe_2O_3).

**
$p < 0.01$ – compared to sham.