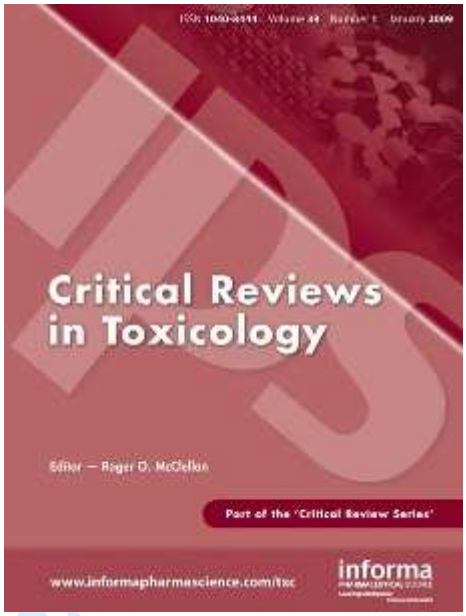

Toxicity of carbon dioxide and its relationship to tobacco smoke

Journal:	*Critical Reviews in Toxicology*
Manuscript ID:	Draft
Manuscript Type:	Review
Date Submitted by the Author:	n/a
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Keywords:	Acute toxicity, chronic toxicity, blood pH, metabolism, cancer, lung, heart, teratogenicity, cigarette, inflammation, central nervous system, hypercapnia, reproduction, PaCO2, carcinogenesis

Toxicity of carbon dioxide and its relationship to tobacco smoke

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Abstract

The toxicity of carbon dioxide has been established for close to a century. A number of animal experiments have explored both acute and long-term toxicity with respect to the lungs, the cardiovascular system and the bladder, showing inflammatory and possible carcinogenic effects. Carbon dioxide also induces malformations and probably reduces fertility. As smokers are exposed to a high level of carbon dioxide (13.5%) that is about 500 times the level in normal air, the aim of this paper is to review the physiological and metabolic mechanisms resulting from CO_2 inhalation and supporting the hypothesis that carbon dioxide plays a major role in the long term toxicity of tobacco smoke.

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Carbon dioxide (CO_2) is naturally present in the atmosphere where its concentration varies from 0.03 to 0.06 % (vol/vol, equivalent to 0.2 mmHg to 0.4 mmHg) (Keeling, 1995). Its regularly increasing concentration contributes to the greenhouse effect (Bertoni, 2004) and the acceleration of global warming (Cox, 2000). The average indoor concentration of CO_2 is 0.08% to 0.1% (National Research Council [NRC], 2008). The maximal acceptable concentration has been defined between 0.5 and 3%, depending on duration of exposure. At normal temperature and pressure, carbon dioxide is an odorless, colorless, and heavier than air gas, with a faintly pungent odor (Shusterman, 1997). CO_2 is widely used in industries, especially in agro-productions for conserving, cooling and medical applications. It is also known to be produced during combustion, putrefaction and fermentation.

In air, carbon dioxide is a very stable and non-flammable compound. As CO_2 is soluble in water, it can react to form carbonic acid (H_2CO_3). Dissolved carbon dioxide in the water undergoes hydration according to the following reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$.This reaction can interfere with the acid-base balance: $pH = pK + \log [HCO_3^-/CO_2]$ (Henderson-Hasselbach equation).

Carbon dioxide is a normal constituent of the human body arising from cellular respiration (National Institute for Occupational Safety and Health [NIOSH], 1976). Carbon dioxide diffuses from cells into the surrounding capillaries and is carried by the blood either bound to hemoglobin or dissolved as carbon dioxide, carbonic acid, or bicarbonate ion (Baggot, 1982). A minor amount of CO_2 can be bound to plasma proteins to form carbamino compounds.

Carbon dioxide is synthesized in the body and its partial pressure under normal conditions in pulmonary capillary blood (almost 7% or 46 mm Hg) is greater than that in alveolar air (6% or 40 mm Hg). The gas is exchanged freely through the alveolar membrane

and is thus released from the lungs by diffusion because of the concentration gradient existing between the blood and the air in the alveoli. Its free diffusion through the lipid cell membranes allows it to be one of the main regulators of intracellular pH acting as a stimulant or a brake in numerous cellular processes. Due to its free diffusion through tissue membranes, the toxicological effects of carbon dioxide appear very rapidly and are mainly observed on the blood pH, lungs, heart and central nervous system.

1. Perturbation of acid/base balance by carbon dioxide

An increase of the partial pressure of CO_2 (pCO_2) delivered to the lungs, i.e., hypercapnia, induces an increase of pCO_2 in the alveoli. Because carbon dioxide freely diffuses through the alveolar membrane to the blood, it results in an increase of the CO_2 tension in arterial blood ($PaCO_2$). This increase in $PaCO_2$ results in turn in an acute or chronic respiratory acidosis.

a- Acute respiratory acidosis

In acute respiratory acidosis, the $PaCO_2$ is elevated above the upper limit of the reference range (i.e., >6.75% or 45 mm Hg) resulting in acidosis (i.e., pH <7.35). This acute hypercapnia can be compensated for in two steps. The initial response is cellular buffering that occurs within minutes to hours (NIOSH, 1976). Cellular buffering elevates plasma bicarbonate (HCO_3^-) only slightly, approximately 1 mEq/L for each 0.15% increase (10 mm Hg) in $PaCO_2$. The second step is renal compensation that occurs over 3-5 days: renal excretion of carbonic acid and bicarbonate reabsorption are increased. In renal compensation, plasma bicarbonate increases 3.5 mEq/L for each increase of 0.15% (10 mm Hg) in $PaCO_2$.

For acute respiratory acidosis, the expected change in pH with respiratory acidosis can be estimated by the following equation: change in pH = 0.008 X (40 - $PaCO_2$) (Smith, 2005). This means that, from a (normal) pH of 7.4 for 6 % $PaCO_2$ and 0.03% CO_2 in the atmosphere, the pH could fall to 6,65 when $PaCO_2$ is increased to 20%. In guinea pigs exposed to 15% CO_2 during 1 hour (acute response), the PaCO2 value was reported to be 17.8 % (119 mmHg) (Schaefer, 1964a).

b- Chronic respiratory acidosis

In chronic respiratory acidosis, the value of the pH is subnormal secondary to renal compensation and an elevated concentration of serum bicarbonate. In rats exposed to 10% or 15% CO_2 during 11 days (chronic acidosis), Carter et al. measured a plasma CO_2 content of 45 and 52 mEq/l respectively and estimated the $PaCO_2$ values at 15% (102 mmHg CO_2) and 22% (148 mmHg CO_2) respectively (Carter, 1959).

In guinea pigs exposed to 15% CO_2 (about 300 time the normal air level) for 73 days (Schaefer, 1961), the uncompensated acidosis period (first variations noticed within one day) is characterized by a decline of extracellular and urine pH, inorganic phosphorus plasma concentration, and an increase of the calcium plasma concentration and urine inorganic phosphorus. During the compensated period, the extracellular pH returns to normal but plasma calcium is still elevated and inorganic phosphorus low level is maintained even after 20 days of exposure. This effect on calcium and inorganic phosphorus was associated with renal calcification after 48 hours of exposure. In rats exposed to 10 or 15% CO_2 for 11 days (Carter, 1959), an increase in urine excretion of ammonia and acidic substances was observed. During the first two days (acute response), the ammonia and titratable acid excretion was almost twice the normal values, and the urine pH value was around 6.2 (10%

CO_2). Potassium and chloride ions were significantly increased during the first days of exposure.

Body adaptation to chronic high carbon dioxide level is dependent of the concentration administered: below 3% CO_2 the compensatory mechanisms occur more slowly. Volunteers were exposed to 1.5% CO2 over a period of 42 days and acid-base balance and changes in electrolyte metabolism were studied (Schaefer, 1964b). During the first 23 days, a slight uncompensated respiratory acidosis was present followed by a compensated acidosis. Interestingly, arterial CO_2 tension increased by 5 mmHg (0.75%) during exposure and remained at this level during the first nine days of recovery in air. Several other studies were performed in men with low levels of increased carbon dioxide exposure (1.5 to 3%) in order to mimic living conditions in submarines or in space. Interestingly, although there were some minor modifications of the pH and serum level of the electrolytes, the experimental conditions were well tolerated (for review see Glatte, 1967a; Glatte, 1967b; Schaefer, 1979).

Guinea pigs were exposed to chronic intermittent high carbon dioxide level (8 hours per day during 7 days, 15% CO_2). While animals exposed to constant CO_2 (15% CO_2 during 7 days) displayed a 3-day-long uncompensated phase and then stabilized (pH: 7.37+/-0.035), animals exposed to intermittent CO_2 could not compensate for the respiratory acidosis, and the pH value was decreased by 0.26 (pH: 7.111+/-0.07) (Schaefer, 1968). Similarly, Schaefer et al (Schaefer, 1970) investigated the acid-base and electrolyte responses to intermittently increased carbon dioxide concentration (concentration increasing up to 3 % CO2, 15 hours/day during 5 days) in human beings. The author reported doubling of urine volume on the fourth and fifth days. This increase in urine volume was accompanied by increases in organic acids, titratable acidity and ammonia, reflecting the elimination of the accumulated carbon dioxide by the kidneys.

Thus chronic carbon dioxide high tension exposure causes a raise of extracellular acidity that is compensated within days (constant 10% or 15%) or weeks (constant 1.5 or 3%). However intermittent exposure to carbon dioxide does not allow the compensation mechanisms to be active.

2. Metabolic effects of carbon dioxide

a- CO_2 implication in cellular metabolism

The effects of carbon dioxide on metabolims have been poorly investigated. Warburg, Posener and Negelein (Warburg, 1924) performed the first work on the metabolic effects of carbon dioxide and they demonstrated the sensitivity of anaerobic glycolysis in a tumor to the concentration of the carbon dioxide-bicarbonate buffer system. In 1943, Craig and Beecher (Craig, 1943) demonstrated that the metabolism in the retina is sensitive to the concentration of the carbon dioxide-bicarbonate buffer system. Increasing the carbon dioxide from 1% to 5% at constant pH increases almost two-fold both glycolysis and cellular respiration. Increasing the carbon dioxide at constant pH from 5% to 20% had no effect on glycolysis, but depressed respiration. This was later confirmed in several studies on different normal and cancer cells (for review see Goldsmith, 1970).

CO_2 is a product of oxidative metabolism but CO_2 and its by-product HCO_3^- is also a substrate for important biochemical reactions occurring, for example, in the mitochondria (Lahiri, 2003). CO_2 takes part into two types of reactions controling respiration in animals: the formation and transport of H+ (by reversible hydration of CO_2 and by formation of carbamates from the NH_2 group of proteins) and the stimulation of metabolism.

HCO_3^- is required in at least three metabolic pathways in the mitochondria of the liver. Mitochondria are impermeable to HCO_3^-, so that the required anion must be provided by the

hydration of CO_2 which can diffuse easily across the membrane. Hydration of CO_2 is the rate limiting factor for these three metabolic pathways. HCO_3^- is involved in the formation of malonyl-CoA (enzyme: acetyl-CoA carboxylase) used for the production of fatty acids components of cell membranes. CO_2 is needed for the conversion of pyruvate to phosphoenolpyruvate during glucogenesis (enzyme: pyruvate carboxylase). Carbon dioxide is also required for the synthesis of carbamoyl phosphate (enzyme: carbamyl phosphate synthetase I). This is known to be the entry in the urea cycle and the regulated reaction of the pyrimidine biosynthesis.

It was demonstrated *in vitro* that the inhibition of a specific liver mitochondrial carbonic anhydrase isoenzyme, the catalyser allowing a rapid conversion of CO_2 into bicarbonate, reduces the formation of glucose, urea and fatty acids in hepatocytes (Forster, 2000). Furthermore, raising the CO_2 concentration (up to approximately 8.5%) increases the carboxylation of ^{13}C labeled pyruvate independently of pH (Ono, 1996).

b- Alteration of in vivo metabolism caused by CO_2

Douglas and al. demonstrated that, in guinea pigs, exposure to 1% CO_2 during six weeks did not alter weight evolution as compared to controls (Douglas, 1979). Schaefer et al (Schaefer, 1971) studied the effect of long-term exposure of guinea pigs to higher tensions of carbon dioxide with respect to several aspects of metabolism. With exposure to 1.5% CO_2, they observed that the guinea pigs lost weight for about 25 days. The animals then start to regain weight but at a slower rate than that of the controls (2.2 g/day versus 4.75 g/day). During long term 3% CO_2 exposure, approximately 35 days are required for the weight of the animals to start increasing above the initial level. During exposure to 15% CO_2, a 10% loss of weight occured during the first two days. At day 20 the rodents started to gain weight for about 20 days to about 50 days.

In vivo CO_2 exposure also affects the expression or activity of certain metabolic enzymes (Schaefer, 1971). Exposure of guinea pigs to 15% CO_2 for seven days results in a striking but transient increase in plasma levels in GOT (glutamic oxaloacetic transaminase) and GPT (glutamic pyruvic transaminase). After seven days of prolonged exposure, the concentrations of these two enzymes return to the initial values. These variations follow the pH changes corresponding to the uncompensated phase of respiratory acidosis. The activity of other serum enzymes such as lactate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and cholinesterase increases significantly during the first three days of exposure (uncompensated phase) and return to control level after seven days.

Histopathology analyses showed that prolonged exposure to CO_2 (3% during 7 days) causes the depletion of glycogen vacuoles and an increase in fat vacuoles in guinea pigs (Schaefer, 1971). After three weeks of exposure to 3% CO_2 and subsequent recovery for one day breathing normal air, glycogen is again synthesized. These functional changes point to important changes in fat metabolism caused by hypercapnia. Acidosis is known to inhibit lipolysis (Poyart, 1968), and one could therefore expect an increase in fat since it would not be easily mobilized. It is noteworthy that both guinea pigs and rats when exposed to even low levels of CO_2 (3%) exhibit similar changes in glycogen and fat vacuolization. That would seem to suggest that modifications of fat metabolism are of special significance in hypercapnia. Lipid accumulation during chronic hypercapnia (15%) shows a specific pattern for different organs. Fat content in muscle is increased only during the first two days, that of lungs during the period from three to seven days, while the lipid content of the liver is greatly elevated throughout the exposure period.

Several *in vitro* or *in vivo* studies have demonstrated that acidosis inhibits lipolytic activity (Triner, 1965; Hollidge-Horvat, 1999). Adrenaline-induced lipolysis and calorigenesis is inhibited in dogs when breathing a mixture of 10% CO_2 and 25% O_2 in N_2

which results in an average pH of 7.0 and an average $PaCO_2$ of 100 mmHg (Nahas, 1965). A study by Longmore et al. showed increased fat synthesis in a perfused liver when the level of CO_2 was raised in the medium (Longmore, 1967). This suggests that another factor adds to the large increase in fat content found in the liver of guinea pigs exposed to 15% CO_2. Similar changes have been observed in both guinea pigs and rats exposed to low levels of CO_2 (3%).

3. Pulmonary toxicity of carbon dioxide

a- Respiratory function

Most toxicological studies have been focused on respiratory damages. Under normal conditions, spontaneous breathing requires feedback controls in which detection of blood gas levels and pH are critical. CO_2 sensing depends on central chemoreceptors (CCRs) located at multiple sites. They are highly sensitive to CO_2, as an evident change in ventilation occurs with an increase in PaCO2 as small as 0.015% (1 mm Hg). Such sensitivity is likely to be attributable to the inherent properties of CO_2/pH sensing molecules (mainly receptors and channels) and their modulation in brainstem neuronal networks. Each of these molecules covers a small range in the whole sensory spectrum. With multiple sensors arranged in parallel, both high sensitivity and broad bandwidth may be achieved (Jiang, 2005).

CO_2 is an asphyxiant and loss of consciousness can occurs when exposed to 30% during one minute, or 10% during 5 to 10 minutes (NAP, 2007). The effects of hypercapnia on the respiratory function appear immediately and at relatively low concentrations (from 1% CO_2). Following exposure to 5% carbon dioxide, there is an increased respiratory minute volume, increased respiratory amplitude and frequency, as well as a decrease in the airway conductance. In monkeys, the respiratory rate increased two-fold until a 10 % carbon dioxide concentration was reached and thereafter decreased until animals died (Stinson, 1970). In a

human study (Schaefer, 1963), 23 healthy men were exposed at a constant level of 1.5 % CO_2 in air for 42 days in a submarine which served as the experimental chamber. Throughout the exposure to CO_2 the respiratory minute volume and alveolar CO_2 tension were increased. During the post-exposure period (9 days), the respiratory minute volume decreased event though the CO_2 tension remained elevated. The authors divided the 42-day exposure period into two parts. The first phase (days 1-23, uncompensated acidosis) was characterized by a significant increased of the alveolar carbon dioxide tension, carbon dioxide excretion and respiratory exchange ratio. The second phase (days 24-42, compensated acidosis) was characterized by an increased excretion of carbon dioxide.

The early acute response to high CO_2 tensions (above 5%) is characterized by an enhanced respiratory volume. This is illustrated by the very rapid (within minutes) of Penh value calculated from plethysmography-measured parameters during the exposure of mice to 5, 10 or 15% (Abolhassani 2009).

b- Acute and chronic lung toxicity

Douglas and al. studied the consequences of chronic exposure (up to 6 weeks) to 1% CO_2 in guinea pigs (Douglas, 1979). They observed an elevation of $PaCO_2$ associated with a metabolic acidosis that reach a maximum at four weeks of exposure and persist even after two weeks of recovery in normal air. Electron microscopy analysis showed changes in cell fine structure of type II alveolar pneumocytes (granular pneumocytes) and lamellar bodies, hyperplasia (cluster of 2-4 cells) and hypertrophia of these cells as compared to control, suggesting an increased activity of theses cells.

Animal studies have indicated that chronic exposure to higher level of CO_2 can cause hyaline membrane formation and atelectasis in guinea pigs and can cause edema in rat lungs. Niemoller and Schaefer (Niemoeller, 1962) exposed guinea pigs and rats to different CO_2 concentrations (from 3 to 15%) during prolonged and continuous exposures (from two days

to six months). Loss of surfactant (complex system of lipids, proteins and lipoproteins which allows the alveoli to remain open throughout the normal cycle of inhalation and exhalation) was associated with hyaline membrane (fibrins, cellular debris lining or filling the alveolar spaces) formation that led to decreased gas exchange, associated with respiratory distress syndrome. Microscopic examination indicated that guinea pigs exposed to 3 and 15% CO_2 developed hyaline membranes (respectively from fourth and first days), while those exposed to 1.5% CO_2 did not, supporting the hypothesis of a threshold for CO_2 induced lung toxicity.

In a follow-up study (Schaefer, 1964a), guinea pigs were exposed to CO_2 and data were gathered from electron microscope studies, surface tension measurements of lung tissue, and additional histochemical studies. These authors identified four phases of pulmonary changes caused by 15% carbon dioxide. The initial phase (6 hours) was marked by uncompensated respiratory acidosis accompanied by pulmonary effusion (edema, congestion, atelectasis and hemorrhage) as well as changes in the lamellar bodies (intracellular stores of surfactant) of the granular (type II) pneumocytes. This period was not associated with hyaline membrane formation. The second phase (6-24 hours) was associated with hyaline membrane formation. During the third phase (days 2-7), the surface tension returned to normal, the pulmonary edema diminished and hyaline membranes disappeared. The final phase was one of recovery despite the fact that the pCO_2 remained elevated.

Recently, Abolhassani demonstrated that inhalation of levels of carbon dioxide above 5 %, for one hour, induced pulmonary inflammation. The authors showed an increase in the secretion of the pro-inflammatory cytokines TNF alpha, interleukin 8, interleukin 6 Mip1-alpha as well as mucin 5AC, a major pulmonary mucus glycoprotein overexpressed during inflammation. This inflammation was caused by the methylation of the C subunit of the phosphatase PP2A, which in turn controls the translocation of the transcription factor NF-κB. Interestingly, complementary *in vitro* experiments do not seem to correlate pH variations

to this inflammation response as IL-8 secretion was not induced in response to acidic pH imposed in the culture medium. These molecular biological findings were confirmed by microscopic examination of the lung. Extensive lung inflammation with infiltration of the parenchyma by lymphocytes and monocytes was observed (Abolhassani, 2009).

Additionaly, Ryu and al. compared the consequences of the exposure of mouse neonates versus adults to 8% CO_2 for two weeks (Ryu, 2010). They showed that CO_2 exposure decreased lung alveolar walls thickness, reduces lung weight and alters lung matrix proteic composition (among them decrease of interstitial collagen) in young mice. In comparison, adult lungs were not affected, which highlighted the sensitivity of young individuals to CO_2 tension variations.

4. Effects of carbon dioxide on cardiovascular function

The first noticeable effects of CO_2 inhalation is an increase in heart rate. For example, exposure to levels of at least 5% CO_2 resulted in the first signs of cardiovascular and vasomotor impacts (cardiac frequency and arterial pressure, peripheric vasodilatation) in humans. The same signs are observed in dogs and monkeys (Stinson, 1970) at concentrations of up to 10 % of the gas. In dogs with left ventricular failure (embolization of the left coronary artery), hypercapnia aggravated the heart failure (increase of the left ventricular end-diastolic pressure, mean right arterial pressure and mean right arterial pressure); however, the pump function of the heart was unchanged (Wexels, 1987). A reversion of the of the central hemodynamics changes was observed when pH is normalized during hypercapnia, meaning that pH, and not $PaCO_2$, was responsible for the observed hemodynamic deterioration.

Schaefer analyzed the heart histopathology of guinea pigs after exposure to 1.5, 3 or 15% CO_2 (Schaefer, 1971). No evidence of permanent myocardial damage was seen either in

animals that expired during the period of acute acidosis or that were sacrified at one day or seven days following initiation of the exposure. However, a small amount of lipid (red O stain) was seen in one animal after one day, and by seven days positive material was seen in five of ten animals. To our knowledge, no similar experimentation was performed to reproduce these data.

5. Effects of carbon dioxide on central nervous system and neuroendocrine function

Carbon dioxide is a key factor in the control of respiration and cerebral circulation. It acts peripherally, both as a vasodilator and as a vasoconstrictor, and is a powerful cerebral vasodilatator.

The majority of the studies reported that chronic low concentration of CO_2 induces low to mild effects: visual impairement occurred at 1% CO_2 and headaches were noticed in the first days of exposure above 2% (National Research Council [NRC], 2007). In general, no specific neurobehavioral changes or adverse effects were reported with level at up to 4% for duration for up to two weeks (Storm, 1974). However, more recent studies showed a decrease in stereoacuity and a decrease in the ability to detect motion with levels above 2.5% (Sun, 1996; Yang, 1997).

At high concentrations, CO_2 exerts a stimulating effect on the central nervous system, while excessive levels exert depressant effects (Lambertsen, 1971). Exposure to 10 % carbon dioxide during approximately 1.5 minutes causes neurologic signs including eye flickering, psychomotor excitation, and myoclonic twitches. At 15%, the same signs were recorded, as well as increased muscle tone, perspiration, flushing, restlessness, dilated pupils, leg flexion and torsion spasms. Apart from excitability, no abnormal behavior has ever been observed after carbon dioxide exposure (psychomotor tests, resolution of problems...) (NIOSH, 1976).

Carbon dioxide induces changes in the secretion of hormones. Continuous exposition (15% CO_2, 7 days) (Schaefer, 1968) stimulates the adrenal gland of guinea pigs. If animals are intermittently exposed to this same concentration of CO_2 (8 hours daily for 7 days), there is an initial fall of pH but no compensation of respiratory acidosis and no changes of the sympathoadrenal responses. From these observations, the authors suggest that the stress response in chronic hypercapnia depends on extracellular and related intracellular changes and is representative of a non-specific pH-dependent effect.

6. Alteration of the reproductive capacity by carbon dioxide

In rats (VanDemark, 1972), carbon dioxide causes degenerative changes of the testes. These modifications depend on both the dose (2.5 %, 5% or 10% carbon dioxide) and the duration of exposure (1 to 8 hours). Major histological effects included tubular disturbances such as sloughing as well as loss of luminal definition (5% during 4 hours), degenerative changes such as streaking and vacuolization (10% during 4 hours). These modifications are reversible, as testes were normal 36 hours after carbon dioxide exposure.

A concentration of 15% chronic CO_2 affects the spermatogenesis of guinea pigs and rats (Schaefer, 1971). The first changes in spermatogenesis are noted after 48 hours. There is a marked decrease in the number of mature spermatozoids. After 3-7 days, multinucleated giant cells are seen. On the other hand, prolonged exposure to low levels (1.5 and 3% CO_2) did not produce any spermatogenic arrest in guinea pigs and rats. Surprisingly, there are no recent data relating carbon dioxide exposure to fertility.

7. Teratogenicity of carbon dioxide

Hypercapnia is teratogenic. Exposition of rats to 6% carbon dioxide (single 24 -period between days 5 and 21 of pregnancy) causes some malformations in the newborn pups (Haring, 1960): cardiac malformations in 24% of the tested animals (7% in control), and skeletal malformations in 11% (0.6% in control). Exposure to higher CO_2 levels (10 %) and consecutive acidosis of neonatal rats promote the retinopathy of prematurity, a potentially blinding eye disorder that primarily affects premature infants (Holmes, 1994 and 1998).

In rabbits (Grote, 1965) exposed to 10-13% carbon dioxide, the newborn pups had vertebral malformations. Furthermore, Nagai A. et al. (Nagai, 1987) examined fetuses from rabbits exposed from day 21 to day 28 of gestation to 8 % CO2 for 8 hours each day. These fetuses weighed less and presented numerous characteristics of increased tissue and cellular maturation of the lung (increased distended lung volumes, increased volume proportion of air spaces, decreased air-space wall, less glycogen and lamellar bodies ...).

Mice exposed to 20% CO_2 for 8 hours on day 10 of gestation produced right-sided postaxial forelimb ectrodactyly in 23% of the offspring. Rather than metabolic acidosis, it would seem that the primary teratogenic factor in hypercapnia is elevated CO_2 tension (low incidence of ectrodactyly associated with NH_4Cl-induced acidosis). Moreover, there is a strong correlation between maternal serum CO_2 content and the incidence of ectrodactyly (Weaver, 1984).

8. Carcinogenic potential of carbon dioxide

a- In vitro alteration of cell fate by CO2 exposure

In 1925, Bauer (Bauer, 1925) exposed chicken tissues to carbon dioxide (concentration level not reported) *in vitro* during 6 to 8 days. The author primarily described the consequences of carbon dioxide on dividing cells, and, in particular the retardation of cell division (in prophase, anaphase and telophase) as well as some alterations of the division

process: “It was noted that the entire equatorial plate moved slowly from one pole to another without a division of the chromosomes”.

In 1927, Mottram (Mottram, 1927) described that the CO_2 tension and/or acidity applied to culture cells control cell activity, in particular cell migration. He also evoked the role that carbon dioxide could play in cancer aetiology. In a follow-up experiment, Mottram cultivated kidneys cells and fibroblasts of young rats with different concentrations of carbon dioxide during 3 days; cells were then fixed and mitosis were counted (Mottram, 1928). From these observations, he deduced that the optimum tension of CO_2 for the cell division in normal cells is the physiological CO_2 tension (6% CO_2) but that cell division occurs at concentrations above (up to 30% CO_2) and below (0 mmHg) this normal tension. Interestingly, whilst counting these mitoses in fibroblasts, many abnormal features were observed in the cultures grown at elevated CO_2 tensions. These features consisted of “an irregular migration of the chromatin towards the centrosomes; some chromatin remained suspended at the equator of the spindle, while other fragments had already migrated to the centrosome. This unusual arrangement was more often than not asymmetrical, a fragment of chromatin being present at one centrosome with none at the other, or more fragments at one centrosome that at the other.[…] It was also observed that the size of the nuclei of undividing cells under high tension of CO_2 was increased, while reduced at low tensions, as compared to nuclei of cells at 40 mmHg (6%) CO_2.” Thus, high carbon dioxide concentration clearly acts as a disrupter of normal cell division processes. The author also noticed that these abnormalities were similar to those observed in cells that had been subjected to X-irradiation, where, “besides fragmentation of the chromatin into fine granules, delay in its migration to the centrosomes occurs, so that whilst some chromatin has moved to the centrosomes, some remains suspended at the equator of the spindle”. Similarly as after X-irradiation, an increase in size of the cell nuclei was observed under high CO_2

concentrations. These observations support the hypothesis of a role of supraphysiological concentrations of carbon dioxide in carcinogenesis via the disruption of cell division processes. Recent data partially complete these earlier findings.

Schuller et al. addressed the mechanisms of cell proliferation in response to nicotine and NNK (nitrosamine 4-(methylnitrosamino)-l-(3-pyridyl)-l-butanone) in normal pulmonary neuroendocrine cells (PNE) cells derived from fetal hamster lung, and in two cell lines derived from human neuroendocrine lung cancers (Schuller, 1994). Their data demonstrated that the mitogenic effects of nicotine and NNK are potentiated by elevated levels of CO_2 (from 8 to 12%) in a concentration dependant manner. Similarly, Merryman demonstrated that a concentration of 10 % CO_2 stimulated the proliferation of small cell lung cancer cells exposed *in vitro*. CO_2 activated the MAP kinase pathway and could be considered as an important messenger molecule in the lung (Merryman, 1997). Interestingly, this article also underlined that chronic nonneoplasic pulmonary diseases (COPD, asthma, emphysema, chronic bronchitis) are characterised by an impaired respiration and an augmentation of carbon dioxide pulmonary tension (7 to 40%) which might promote lung cancer development.

These articles supports the hypothesis that high carbon dioxide tension might promote cancer development.

b- In vivo carcinogenicity of carbon dioxide

The following articles describe the carcinogenic effects of CO2 *in vivo*. It should be noted, however, that for most of them, the concentrations of carbon dioxide used were very high.

In a transplantation experiment, skin autografts were exposed *in vitro* before transplantation during 48 hours to 45 to 48% CO_2 in air (control: room air culture). Although

some malignant lymphoma were observed in host animals using untreated autografts, the lymphoma incidence was highest in the recipients of the CO_2-treated grafts. Other abnormalities of the reticuloendothelial system were noted: proliferation of lymph follicles into irregular masses of pleomorphic cells, hyperplasia with concommitant atrophy of the lymphoid tissue, and replacement of the lymph follicles by malignant lymphoid cells (Goldsmith, 1975).

The long-term clinical effects of high CO_2 tensions on various normal tissues in mice have been investigated (Goldsmith, 1980). Different tissues were exposed *in vitro* to a high CO_2 proportion in air (45% CO_2) before transplantation into syngeneic or autologous hosts or, in a second protocol, intraperitoneal tissues were exposed *in vivo* to CO_2-infusion (99.99%), thus avoiding graft-host interactions. In the autologous grafts, pretreatment by intraperitoneal CO_2-infusion induced lymphoma (60% incidence), air-infusion did not. Non-lymphoid grafts exposed *in vitro* to elevated CO_2 induced only lymphoid malignancies. But non-lymphoid tissues exposed *in vivo* to elevated CO_2 developed tumors of other tissues, such as lung tumor, in addition to lymphoid malignancies. In fact, the spontaneous pulmonary adenocarcinoma incidence doubles in the mice exposed to intraperitoneal CO_2. The same morphological lymphoid abnormalities occurred in all lymphoma-developing animals in these three experimental models: hyperplasia in the splenic T-cell areas appeared most frequently (70-75 % incidence), whereas atrophy in T-cell areas of the lymp nodes and B-cell areas hyperactivity were far less frequent.

In a mouse model for multiple laparoscopies, intraperitoneal insufflations of approximately 3.5 ml of CO_2 were given daily to three groups of BALB/c mice for 11, 20, and 32 consecutive days (control: air insufflation). Proliferation of splenic T-lymphocytes (doubling of the T-cells spleen percentage) was an early, but transitory, immunologic reaction in the spleen to intraperitoneal CO_2 insufflation. This was correlated to the late

occurrence of a high incidence of malignant lymphoma (approximately 60%). The long-term survivors of CO_2 insufflation also developed a wide spectrum of malignancies that were not of lymphoid origin, specifically adenocarcinoma in various organs: lung, kidney, adrenals, ovary, gastrointestinal tract and salivary gland (Goldsmith, 1981).

The effects of high concentrations of CO_2 on experimental murine neuroblastoma tumors have also been studied (West, 1978). The local growth of this neuroblastoma model was not affected by concentrations of 76% and 55% of CO_2 applied for 10 and 30 minutes. Although, the tumor bearing animals exposed to different CO_2 concentrations tended to develop metastases more frequently than the control groups.

9. Does carbon dioxide contribute to cigarette smoke toxicity?

Below its immediatly lethal concentration, carbon dioxide has long been considered as a neutral compound for the body. However, recent studies raised interest on carbon dioxide in relationship with chronic and/or intermittent long term exposure conditions that might induce pathologic states, in particular nasal inflammation (Buron, 2009; Hacquermand, 2010) and pulmonary inflammation (Krohn, 2003; Abolhassani, 2009).

There are various situations when pCO_2 can rise in the inhaled air. First, during professional exposures such as recurrent manipulation of dry ice, food and floral preservation, wearing of mask, spacecraft, aircraft, submarine, mask, altitude, exposure to combustion gas (Roberge, 2010; NRC, 2007; NRC, 2008). Second during pathological exposures such as sleep apnea, pulmonary diseases (e.g. COPD, asthma, emphysema, chronic bronchitis) (Windisch, 2005).

Smoking, as every combustion produces CO_2 (about 13% in the mainstream smoke). And, even if the exposure is quite short, it is also repetitive and regular. Our hypothesis is that carbon dioxide cigarette smoke content might be implicated in smokers pathologies.

Like CO_2, exposure to tobacco causes both acute and chronic lung inflammation (BPCO). In a recent paper, we demonstrated that the acute toxicity of cigarette smoke is due to carbon dioxide inhalation. Using a KOH filter, we were able to selectively trap bicarbonates (and thus CO_2) but not oxygen or particles. The decrease in carbon dioxide concentration results in the almost complete disappearance of the inflammatory syndrome caused by cigarette smoking and expressed by the mice (Schwartz, 2010). The precancerisation events described with *in vitro* exposure (cf. § 8) and the concept of cancer as an inflammation-based disease lead us to think that carbon dioxide might be a major contributor of lung carcinogenesis.

Bladder cancer is one of the most common human cancers, consisting about 6% to 2% of all cancers among men and women, respectively. The exact mechanism by which smoking increases the incidence of this malignancy is not known but some authors discuss the role that inflammation might play (Burin, 1995). As hypercapnia, smoking tends to increase urine acidity (Fix, 1986; Dales, 1978). Wald et al measured urinary pH in 145 cigarettes smokers and found that 70% of the smokers have a pH value below 7 (Wald, 1984). Furthermore, there is evidence that acidic urine has a negative influence on urothelial cell DNA adducts levels in humans who have been exposed to benzidine, and the aromatic amines derived from cigarette smoke. Individuals with urine pH lower than 6 had 10-fold higher DNA adduct levels compared to subjects with urine pH 7 or greater (Rothman, 1997). Hence, the acidity of urine caused by smoking might be an important susceptibility factor for the development of bladder cancer.

Another aspect of carbon dioxide potential role in carcinogenesis is its implication in cell metabolism. As previously described in §2, CO_2 is a key regulator of three metabolic reactions. It can be presume that enhancing CO_2 levels in the cells will displace the equilibrium of these reactions and favor the production of building elements for new cells.

Smoking is known to be carcinogenic. Smoking induces a wide array of cancers such as cancer of the lung, head and neck, esophagus and the bladder. The toxicity of carbon dioxide has been established for close to a century. It is possible that its role in the epidemic of tobacco related illnesses has been overlooked. Carbon dioxide could also interact with tar making the animal more susceptible to its toxicity. There are no clear data demonstrating the carcinogenicity of carbon dioxide, but short term exposures of cells or rodents suggest that it is highly likely, a strong probability that further studies would confirm.

Acknowledgments

We acknowledge the help of Jean-Marc Steyaert and Edward Sanders. This work was funded by Biorébus.

Competing interests

AG is an employee of Biorébus. The other authors declare that they have no competing interests.

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