Abstract

Introduction: Results from clinical observations have demonstrated that percutaneous infiltration of carbon dioxide improves the appearance of the skin in adjacent areas. No studies have been found in the literature that showed evidence of histological changes caused by carbon dioxide injections.

Objectives and Methods: A blind cross-sectional pilot study was performed in the Departments of Pharmacology and Morphology of the Federal University of Ceará, with the aim of histologically investigating whether intradermal and/or subcutaneous injection of medicinal carbon dioxide would increase collagen turnover in rats. Ten male Wistar rats were used, aged 3 months (2 animals) and 14 months (8 animals). The 2 younger rats were used as controls. Four of the older rats received injections of saline solution (0.9%), and were also considered to be controls. In the remaining 4, carbon dioxide was injected into the subcutaneous cellular tissue and intradermally. Biopsy samples were collected before and after treatment with carbon dioxide.

Results: Collagen turnover increased in the treated animals in comparison with the controls. Compression of collagen bundles in the tissue samples where intradermal injection was used was more intense than in the subcutaneous treatment. The histological characteristics of the samples with carbon dioxide injected intradermally were similar to the characteristics of the younger rats (controls).

Conclusions: The results obtained corroborate clinical observations of aesthetic improvements in the facial skin with carbon dioxide injections. Future research should address the comparison between intradermal and subcutaneous injections, the volume of gas used, and the frequency of treatment sessions.

Introduction

Human tissue changes with age. In the skin, these modifications are more easily recognized.1,2 The modifications to the collagen and elastin system cause wrinkles, atrophy, grooves, ptosis, and laxity. These are the most evident signs of old skin,3,4 which can result in significant psychological problems for individuals. There is increasing interest in therapeutic resources for solving such problems.5 For this reason, the morphological and structural changes to the dermis related to aging are constantly being studied.

Many authors have shown that there are beneficial effects from subcutaneous carbon dioxide (CO₂) therapy for several clinical conditions.7-20 Intradermal injection of CO₂ has been studied recently, especially because of the clinically favorable results observed, its low cost, and safety.8 However, there is a lack of scientific evidence regarding the histological modifications that occur with the intradermal injection of CO₂.

Dermis Aging

Senile dermal modifications occur mainly in the extracellular matrix (ECM), a substance within the extracellular space that provides the supporting structure to cells, as well as resistance to compression and stretching. It is also the medium through which nutrients are offered to cells and into which cellular excreta are ejected.21,22 The extracellular matrix is composed of fluid and fibrous components. One of the most common fluid components of the ECM is hyaluronic acid. In combination with certain proteins, this becomes a highly viscous and hydrophilic mucopolysaccharide. Elastin, fibronectin, and collagen are the basic elements of the fibrous component of the dermis. Collagen is the most abundant of these, and it is the main element of human skin. It is responsible for maintaining the structural integrity of the skin by joining cells to other cells and the ECM.

Collagen is an insoluble protein that forms fibers, which in turn join together in bundles. Fibroblasts secrete the precursors of collagen, protocollagen types 1 and 3. Aging brings a decrease in the number of skin fibroblasts and, at the same time, an increase in collagenases, which catalyze the degradation of collagen. It also causes a reduction in the number of cells and vessels and this, together with biochemical changes in collagen constitution, leads to thinner collagen fibers. The collagen content per unit of skin area decreases by 1% per year over the course of adult life and collagen fibers become disorganized, less compact and more granular; collagen becomes more rigid and less elastic. The consequence of this is the loss of dermal volume in older individuals.
Some other clinical manifestations of aging, such as wrinkles, grooves, and atrophy, as well as changes in facial format, are due to the new architecture of the skin’s conjunctive tissue. Aging is a progressive, universal process that is subject to environmental, genetic, and hormonal factors. Among the environmental factors associated with skin aging, ultraviolet radiation is the most important.

Antiaging Treatments
Many treatments have been used to reduce the effects of the skin aging process. Antiaging treatments have included tretinoin (retinoic acid), antioxidants like vitamins C and E, phytoestrogens, dimethylaminoethanol, and many others in the form of creams, gels, and lotions. They can be used prophylactically, as adjuvant therapies in some circumstances, or as the sole treatment. Invasive techniques are also available and these seem to be more efficient, including chemical or laser peeling, dermal filling with various substances or with solid implants, botulinum toxin, and many plastic surgery techniques. More recently, hormonal modulation has been proposed as a prophylactic method, and CO2 therapy is also being used as a promising technique against skin aging.

Carbon Dioxide Therapy
Subcutaneous administration of CO2 is popularly known as carboxitherapy. Subcutaneous infiltration is a recent innovation in medicine, but the administration of CO2 began in France in the 1930s, where peripheral arteriopathy was treated with CO2 gas. The publication of studies on CO2 therapy began in the 1950s, but most of the work papers were published between 1985 and 2002.

The main indications for CO2 therapy are peripheral arteriopathy, acrocyanotic syndrome, venous insufficiency and foot and leg ulcers, adipose tissue accumulation, symmetrical multiple lipomatosis, and others.

In the 1990s, video laparoscopy was used for injecting quantities of more than 3 liters of CO2 at rates reaching 1 liter per minute without adverse effects. Thus, the use of this gas, which is formed naturally by the body at rest and during exercise, is safe. Moreover, it is eliminated within a short time. Adjacent to the areas where CO2 was injected, surgeons noticed reductions in the quantities of adipose tissue and fibrous edema. This prompted new clinical research using subcutaneous CO2 to treat adiposity and aging.

A histological study on patients treated with CO2 injections for adiposity showed that the treatment did not lead to any damage affecting the connective tissue, vascular bed, or nerve structures. The recent use of CO2 in angiographic procedures has proven the safety of this cheap and nonallergenic gas. Carbon dioxide does not provoke embolism even with bolus injections of 100 ml, and a continuous flux of 20 to 30 ml/second does not induce adverse reactions. Local or systemic complications have not been reported in the literature. The only side effects are pain, hematomas caused by the puncture, and a crepitation sensation caused by the small local subcutaneous emphysema, which disappears within 30 minutes. Carbon dioxide injections are contraindicated for phlebitis, cardiac/respiratory insufficiency, renal/hepatic insufficiency, severe arterial hypertension, and pregnancy.

Carbon dioxide provokes local vasodilation and increased regional blood flow and oxygen pressure; there is a reduction in the affinity of hemoglobin for oxygen, thereby resulting in more availability of oxygen for the tissue. Many authors have observed this phenomenon after subcutaneous injection of CO2, using many different examination techniques.

Objective
This study was designed to investigate whether CO2 injection into the dermis of Wistar rats can improve collagen turnover induced by intradermal injection of carbon dioxide in rats.
INCREASE IN COLLAGEN TURNOVER INDUCED BY INTRADERMAL INJECTION OF CARBON DIOXIDE IN RATS

Methods
This pilot study was a blind, interventional, cross-sectional study, with qualitative analysis of the results, carried out in the Pharmacology and Morphology Departments of the Federal University of Ceará (UFC), from February to March in 2006. The experimental study followed the principles for research using animals: the subjects had full access to water and food before and after the procedure and their life cycles (diurnal and nocturnal) were respected. The study was approved by the Ethics Committee for Animal Research of this university. The animals were provided by the Pharmacology Department of UFC.

Ten male Wistar rats, born from the same mother and father, 2 of them young (3 months old) and 8 of them old (14 months old), were used. The rats were subjected to a skin biopsy 1 day before the procedure and those presenting with dermatological or subcutaneous problems were excluded. After either subcutaneous or intradermal CO2 infiltration, the morphological alterations (quantity and arrangement) to the dermal collagen fibers were evaluated (Figure 1a).

Preprocedure Biopsy
After undergoing anesthesia (by means of inhaling ether from a cotton ball inside a ventilated chamber), all 10 animals (young and old) were shaved and skin samples were collected by punch biopsy (1 mm diameter in the right posterior flank, 3 cm distally from the femoral joint).

Procedure
One day after the first biopsy, areas of 1 cm² were shaved and marked out on the skin of each of the 8 14-month-old rats (Group T=treated): 1 area on the left anterior flank and the other on the right anterior flank (3 cm posteriorly to the scapulohumeral joint).

A disposable needle (30 G 1/2) was attached to the Carbtek carboxitherapy equipment (Estek, São Paulo, Brazil) by means of an appropriate device with an anti-reflux filter. The manometer pressure was calibrated to 15 mmHg, with a flow velocity of 20 mmHg.

For 4 of the 14-month-old rats, CO2 infiltration was performed intradermally (GTI) on the right side. This was done by means of introducing only the bevel of the needle into 1 of the vertices of the square that was marked out, until the intradermal infiltration of CO2 caused a white papule in the center of the marked square. On the left side of the same rats, the injection was subcutaneous (GTS), with 3 mm of the needle introduced from 1 of the vertices of the square marked out, until the gas volume was enough to cause skin distension in the marked area. The point of the needle was kept in the corner of the square to preserve the central area for puncture biopsy (Figure 1b).

For the other 4 14-month-old rats (control group), injection of saline solution (0.9%) into the subcutaneous tissue caused distension of the areas marked out with squares.

Biopsy and Histological Analysis
On the sixth day after the CO2 infiltration therapy, the 8 14-month-old rats (4 treated and 4 controls) were again anesthetized with ether and the total skin thickness was biopsied using a 1-mm punch at the center of the 1-cm squares. Both the preprocedure biopsy specimens (from all 10 animals) and the biopsy specimens collected after the procedure (from the treatment group) were prepared for histological analysis.

The specimens were fixed in formaldehyde and dehydrated in a series of increasing concentrations of ethyl alcohol. After this, they were diaphanized, embedded in paraffin blocks and cut into 4-mm sections. The specimens were then subjected to Mallory’s trichrome staining, in which collagen synthesis

Figure 2. Photomicrograph of Wistar rat dermis biopsy a) before and b) after subcutaneous CO2 infiltration, with less dispersed and more numerous collagen fibers.
is shown in blue. The analysis was performed by an experienced pathologist using a binocular optical microscope with millimeter scale (Leitz Periplan GF 100X, Wetzlar, Germany). Digital photos of the histological slices were taken (Collpix camera, Nikon, Japan). The pathologist was not aware of the identification of each animal specimen (ie, whether it was from the treated or the control group).

**Results**

No side effects were observed after the injection of CO2 or saline solution.

Histological analysis showed intense collagen turnover in the skin samples of the animals treated with CO2, especially in those with intradermal treatment, in comparison with the animals that had only had saline solution injected in the dermis. Collagen synthesis was shown by Mallory's staining. The collagen fibers were less dispersed (Figure 2) following both subcutaneous and intradermal CO2 infiltration. Intradermal injection made the collagen fibers more compact than did subcutaneous injection. After intradermal infiltration, the collagen arrangement in the dermis of the old animals in this study was similar to that of the 2 young rats (Figure 3).

Subcutaneous saline solution injection into the control group rats caused greater collagen fragmentation, comparing the preprocedure biopsy with the biopsy after the procedure in the control group (Figure 4).

**Discussion**

This study showed visible compacting of collagen fibers following CO2 infiltration, especially among the skin samples with intradermal infiltration. Oria et al (2003), in a study using cadavers, had already demonstrated that aging caused the collagen bundles to disperse, since collagen fibers are more compact in young humans.47 In fact, after CO2 injection, the arrangement of the collagen in the dermis of the older rats in this study was similar to that of the younger animals.

Figure 3. Photomicrograph of rat dermis biopsy after intradermal CO2 infiltration in a) 14-month-old Wistar rat and b) 3-month-old rat.

Figure 4. Photomicrograph of rat dermis biopsy after saline solution infiltration in a 14-month-old Wistar rat (control).
Many authors have shown that injection of CO₂ into the subcutaneous layer leads to an increase in the blood flow to the area. This blood flow increase may lead to neoangiogenesis in cases of chronic exposure to CO₂ injection, and could partially explain the more intense collagen synthesis adjacent to the treated area in this study. In a randomized, controlled study by Brandi et al, subcutaneous injections of CO₂ to treat adiposity caused an increase in oxygen pressure, and skin thickness, and a decrease in the circumference of the treated areas. A histological examination showed that the adipocyte membrane was broken, and that connective tissue, vessels, and nerves were preserved. Other studies are necessary to confirm these findings and the exact mechanism of action. It may be necessary to use hyaluronic acid together with CO₂ therapy to enhance results.

Elastic fibers break during the process of aging, thereby causing harm to dermal tissue and flaccidity. Recent clinical observations have shown that CO₂ therapy can be used to treat laxity and that the intradermal layer is the best place to perform gas injection, as demonstrated in this study. It remains to be seen whether this subtle but important change in the place of injection is capable of inducing elastic fiber production (Figures 2 and 3). The present study did not aim to evaluate the quantity of collagen fibers nor the volume of gas to be injected, but simply to investigate whether CO₂ injection could increase collagen turnover. Other work in the scientific literature with this objective was found. Brandi et al (2001) showed that there was an evident increase in the dermal thickness of patients treated for adipose accumulations using subcutaneous CO₂ injections. Lipolysis may, in such cases, be associated with increased collagen turnover.

Randomized trials may help to increase knowledge and establish scientific parameters for the use of CO₂ therapy. Future research should use a larger number of animals (possibly rabbits, whose skin is more similar to human skin) for studying CO₂ therapy for aesthetic problems relating to aging skin, and try to standardize. The frequency of CO₂ injection sessions and the number of sessions needed, the spacing between the injection points on the skin, and the volume of gas to be injected.

Conclusions

The data from this pilot study suggested that CO₂ injection caused increased collagen turnover in rats, which was more pronounced with intradermal injection. These results support the clinical observation of reductions in wrinkles consequent to CO₂ therapy and also establish that intradermal injection is better than subcutaneous injection.

References