

Effect of Changes in Inspired Oxygen Tension on Wound Metabolism

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This work was prompted by earlier findings of the beneficial effect of increased oxygen supply on wound healing. Enzyme activities in the limiting step of glycolysis, citric acid cycle and pentose phosphate cycle were determined in cellulose sponge implants of rats chronically breathing 12% O₂, air or 55% O₂. Respiratory gas tensions and concentrations of pyruvate and lactate were measured in wound fluid aspirated from the implants. Significant portions of repair tissue exist in conditions of extremely low oxygen tension. Probably because all added oxygen is readily consumed, the wound fluid PO₂ increased only slightly in hyperoxic environment. The wound PCO₂ increased in parallel with the inspired PO₂, probably due to enhanced production of carbon dioxide. Hyperoxia shifted the wound metabolism from anaerobic towards aerobic glycolysis. This occurred concurrently with activation of citric acid cycle. Succinic dehydrogenase, a linking enzyme between citric acid cycle and electron transfer chain, also increased with increasing oxygen tension. This oxygen-induced metabolic change has been previously observed in many other tissues.

OXYGEN METABOLISM of healing tissues gained practical importance after it was shown that repair processes respond to changes in arterial oxygen tension. An improvement in the supply of available oxygen promotes healing in both soft tissue wounds and fractures.^{5,9,16,23,27} Conversely, reduction in oxygen tension impedes repair processes.^{5,8,9,17}

The supply of oxygen at the healing edge is extremely precarious. Oxygen gradients are steep between the capillary and the healing tissue a few microns away and significant portions of any injured tissue exist in conditions of low oxygen tensions.^{13,18,22} Obviously, this is not optimal for healing since the rate of collagen

accumulation has been shown to be directly proportional to the amount of oxygen delivered to the wound.^{5,9,23}

Although local dynamics of extracellular oxygen in wounds are well described, knowledge of the cellular mechanism is still sparse. During prolonged hyperoxia fibroblasts do not increase in number but they possess increased amounts of ribonucleic acid and may have, therefore, a greater capacity for protein synthesis.^{9,10} In normal wounds the phase of fibroblast proliferation is characterized by a relatively high activity of pentose phosphate cycle, whereas glycolysis dominates during the synthesis of collagen.⁷ In hyperoxia collagen synthesis has increased in parallel with the utilization of glucose.⁹

Despite the accumulating evidence it has been difficult for many to accept that conditions in wounds may not be optimal for healing and that "super-normal" healing can be induced by merely elevating the oxygen supply to the repair area. Therefore, this study was undertaken to test the energy metabolism of wound cells as affected by chronic exposure to normal, high, and low oxygen tensions in an experimental model, the implanted cellulose sponge cylinder; to investigate the effects of oxygen supply on granulation tissue enzymes in limiting steps of glycolysis, pentose phosphate cycle and citric acid cycle; and to measure the changes of respiratory gases, pyruvate and lactate in the wound produced by chronic exposure to low and high ambient oxygen tensions.

Methods

Viscose cellulose sponge (Visella, Säteri Oy, Valkeakoski, Finland) was used as an inductive matrix

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for repair tissue, as described by Niinikoski and coworkers.^{11,12} The material was cut into cylindrical pieces, 50 mm long and 10 mm in diameter, and a tunnel 3 mm in diameter was made through the center of the sponge. Silicone rubber discs (10 mm in diameter and 2 mm thick) were stitched onto both ends of the sponge to create a stable dead space. This dead space fills with wound fluid which can be removed and analyzed at will. The cylinders were sterilized by boiling for 30 min in physiological saline solution prior to implantation. An incision, 3 cm long, was made in the dorsal midline of male Wistar rats weighing 160–180 gm and two cylinders were implanted longitudinally under the skin, one on each flank. The skin wound was closed with silk sutures. Implantations were performed with ether anesthesia under clean but not strictly sterile conditions.

Thirty rats were studied in three groups of ten. Immediately after implantation the first group was exposed to 12% O₂, the second group to 55% O₂, and the control group to air. All the animals were housed in transparent Perspex containers, volume about 23 l, the floors of which were covered with wood shavings. Five rats were kept in each container.

Gases were supplied to the containers at atmospheric pressure and the flow rates were adjusted to 5 l per minute. The gas concentrations inside the chambers were checked twice a week. Oxygen concentrations in the chambers varied between 11.9 and 12.4% in the hypoxic group, between 51.6 and 59.8% in the hyperoxic group and between 19.7 and 20.8% in the air group. The CO₂ level remained below 0.5%. The relative humidity was between 70 and 85% and the temperature approximately 1 C above room temperature (about 20 C). The chambers were opened once a day for approximately 5 minutes for feeding and cleaning. Drinking water and food pellets were supplied *ad lib*.

On the 7th, 11th and 16th day after implantation, samples of wound fluid were aspirated from the granulomas as described by Niinikoski and coworkers.^{11,12} Without interruption of exposure to different oxygen tensions 2–3 ml of fluid were collected from each implant using sterile hypodermic needles. The PO₂ of the wound fluid was measured in a Clark-type oxygen electrode and the PCO₂ was determined in a Severinghaus carbon dioxide electrode at 37 C. Zero adjustment of the O₂ electrode was obtained with gaseous nitrogen and the calibration took place with aerated saline, PO₂ 150 mm Hg. The CO₂ electrode was calibrated with two moistened gas mixtures of known carbon dioxide tensions (26 and 57 mm Hg).

For determinations of wound fluid pyruvate and lactate, 1 ml aliquot of fluid was deproteinized by adding an equal volume of 1.0 M perchloric acid. Pyruvate and lactate were determined by the standard enzymatic

methods (C. P. Boehringer & Soehne GmbH, Germany; Biochemical Test Combination) using a Beckman DU spectrophotometer.

Seventeen days after implantation the rats were decapitated. The implants were removed and frozen immediately to –20 C. For the measurements of enzyme activities each implant was homogenized in 15 ml of ice-cold 0.9% sodium chloride solution for 4–5 sec. in an Ultra-Turrax TP 18/2N homogenizer (Janke & Kunkel KG, Germany) and the mixture was centrifuged for 15 min. at 600 G. An aliquot of 1.1 ml of the supernatant was used for the measurements of total enzyme activities,²⁴ and the amount of DNA in the implant was determined from the remaining supernatant and the sediment.²⁰

The enzyme activities were calculated as international units per milligram of DNA to demonstrate the oxygen-induced metabolic changes at cellular level. All enzymatic determinations were carried out according to original methods. Hexokinase,²¹ pyruvate kinase,³ and lactate dehydrogenase⁶ were used to represent the enzymes of glycolytic pathway. The activities of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase reflected the function of pentose phosphate cycle.² NADP dependent isocitrate dehydrogenase,¹⁴ malate dehydrogenase¹⁵ and succinic dehydrogenase⁴ represented the enzymes of citric acid cycle.

The enzyme activities were measured at 30 C using a thermostatically controlled Unicam SP 800 recording spectrometer. For the determination of succinate dehydrogenase 600 μ m absorption maximum of 2,4-dichlorophenolindophenol was used. The other methods were based on the measurement of 340 μ m absorption band of NADH or NADPH.

Results

All rats gained weight during the study (Fig. 1). In 12% O₂ the increase in rat weight was only 50% of that in the other groups. No deaths occurred during the exposure and all implants remained uninfected.

In the early phases of healing the dead space PO₂ was almost zero (Fig. 2). On the 16th day the highest wound oxygen tensions were observed in rats breathing 55% O₂ and the lowest tensions in rats breathing 12% O₂. However, the differences were not statistically significant.

The higher the inhaled oxygen concentration the higher the wound fluid PCO₂ (Fig. 3). Seven days after implantation the mean PCO₂ was 121 mm Hg in 55% O₂, 96 mm Hg in the controls and 84 mm Hg in 12% O₂ ($p < 0.001$; analysis of variance). On the 11th day there was an overall decrease in PCO₂ values and on the 16th day the carbon dioxide tensions almost reached the 7-day values.

As healing progressed the pyruvate concentrations

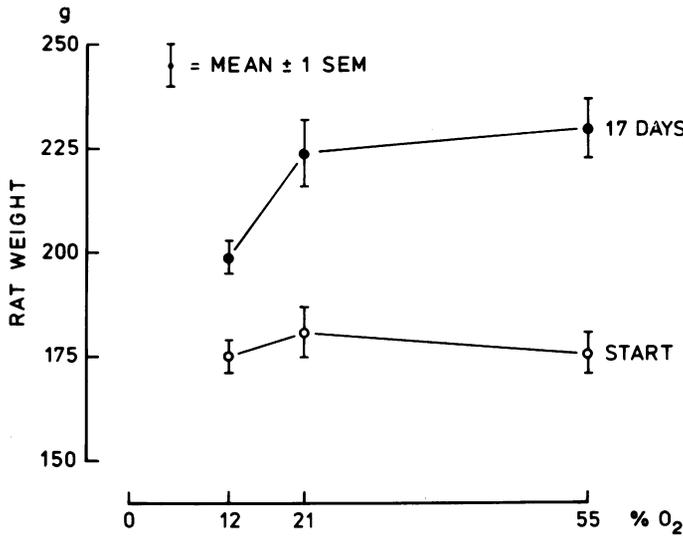


FIG. 1. Graph depicting the weight gain of rats during 17-day exposure to various oxygen concentrations.

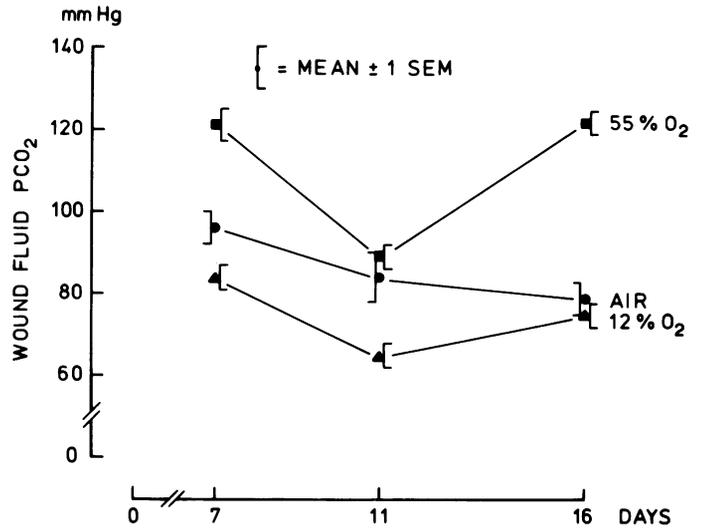


FIG. 3. Effect of changes in inspired oxygen tension on wound fluid PCO₂.

increased, Fig. 4). In hypoxia the values remained significantly below the control level ($p < 0.05$ for 16-day wounds: t-test).

Changes in inspired oxygen were clearly reflected in the concentrations of lactate (Fig. 5) which declined in hyperoxia and increased in hypoxia ($p < 0.01$ for 11- and 16-day wounds, analysis of variance).

Lactate/pyruvate ratios usually declined as healing progressed (Fig. 6). However, in 12% O₂ the values remained elevated throughout the observation period ($p < 0.05$ for 11-day wounds and $p < 0.001$ for 16-day wounds; t-test).

Seventeen days after implantation the amounts of DNA showed no marked changes between the three

groups and the mean values varied from 8.2 to 8.7 mg per implant (Fig. 7).

Increase in the ambient oxygen tension caused a progressive rise in the activity of pyruvic kinase and a decline in lactate dehydrogenase (Fig. 8). In the analysis of variance these changes were statistically significant ($p < 0.001$ for pyruvic kinase and $p < 0.05$ for lactate dehydrogenase). Hexokinase activities were slightly decreased in hypoxia.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed no marked changes between various oxygen concentrations (Fig. 9).

As shown in Fig. 10, isocitrate dehydrogenase and

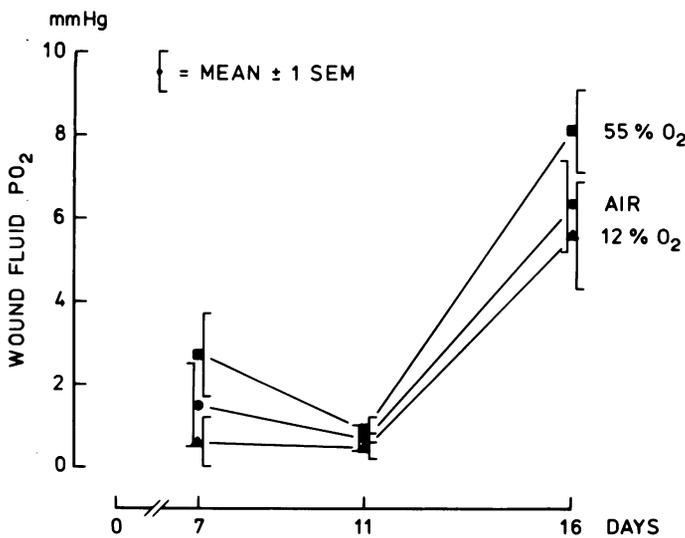


FIG. 2. Effect of changes in inspired oxygen tension on wound fluid PO₂.

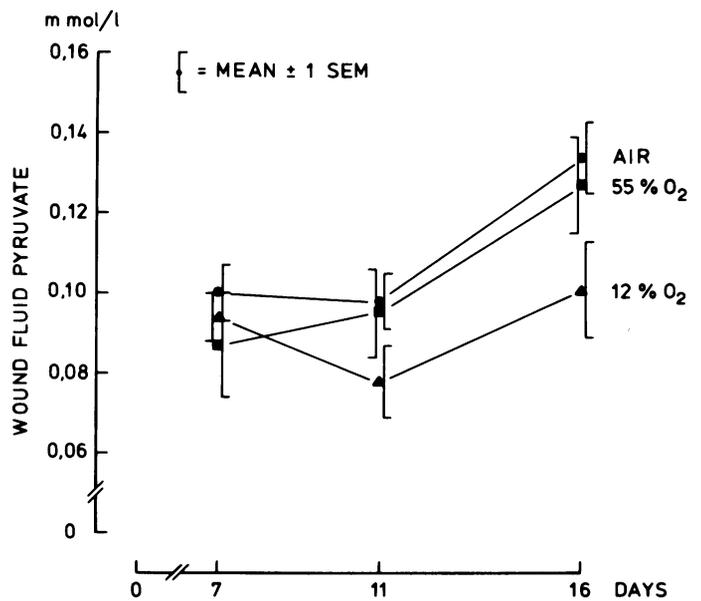


FIG. 4. Effect of changes in inspired oxygen tension on wound fluid pyruvate.

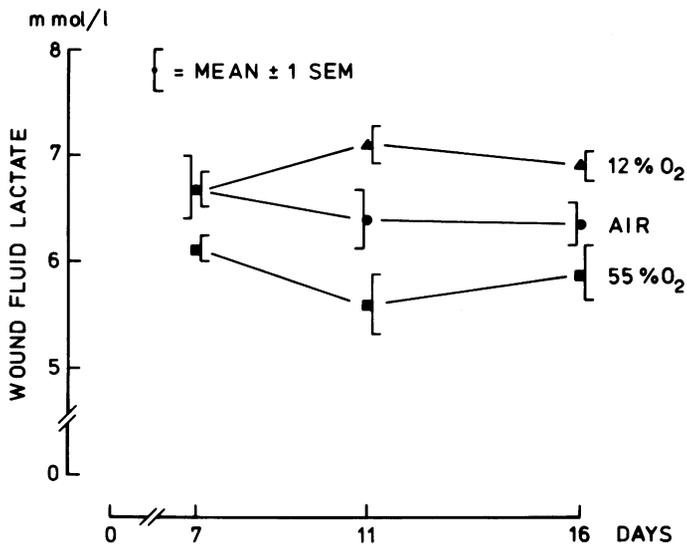


FIG. 5. Effect of changes in inspired oxygen tension on wound fluid lactate.

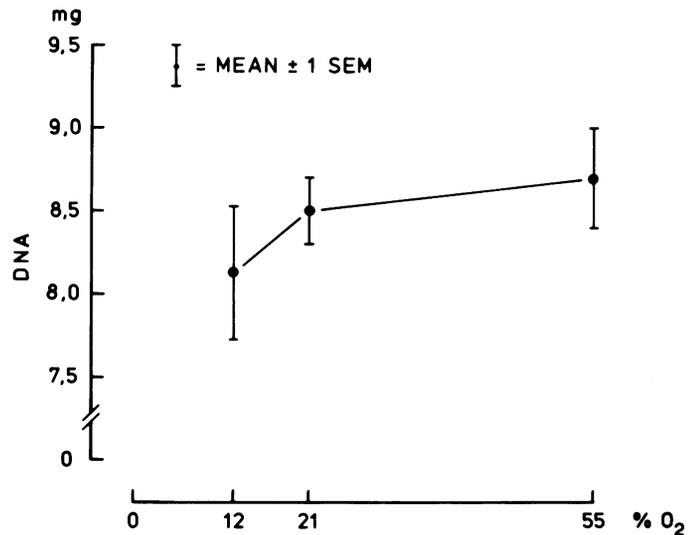


FIG. 7. Effect of changes in inspired oxygen tension on the amount of DNA in experimental granulation tissue. The determinations were carried out 17 days postimplantation.

malate dehydrogenase remained unchanged whereas succinic dehydrogenase increased in parallel with the inspired oxygen tension ($p < 0.001$; analysis of variance).

Discussion

Wound Model

The biochemistry of the wound model used in the present study has been thoroughly investigated.^{1,7,9,25} The composition of wound fluid filling the central dead space has also been extensively studied.^{11,12,19,26}

Effect of Oxygen on Wound Healing

Niinikoski⁹ has shown that accumulation of collagen and the amount of ribonucleic acid produced in cellulose sponges in rats are decreased by hypoxia and increased

by hyperoxia, reaching a peak at 70% ambient oxygen at one atmosphere pressure and decreasing again during constant exposure to higher oxygen tensions. Wound tensile strength also varied according to ambient oxygen tension so that 70% O₂ increased the tensile strength by 30%. The beneficial effect of oxygen on wound healing was confirmed by Hunt and his colleagues.^{5,23} They found that collagen synthesis in healing wounds increased above normal in hyperoxia and that collagen accumulation is roughly proportional to the arterial blood PO₂ at nontoxic ambient oxygen tensions.

Wound PO₂

The mechanism of the effect of hyperoxia has been suggested by observations on wound gases. It has been demonstrated that when arterial PO₂ is elevated the mean capillary PO₂ rises, more oxygen reaches wound tissue and the arterial capillary-tissue oxygen gradient increases.^{5,13,18,22} According to Hunt and Pai⁵ the only way to explain the increased tissue gradient is that cells at the advancing edge of granulation tissue are not consuming as much oxygen as they can. If more oxygen is presented to the wound, more is used.

Niinikoski and Kulonen^{9,10} demonstrated that the ratio RNA/DNA of wound cells increases during chronic hyperoxia. This suggests that the fibroblasts can increase their synthetic apparatus if added oxygen is available. Hunt and Pai⁵ reasoned that the change in the ratio RNA/DNA takes time and predicts that the immediate increment in wound PO₂ after a few hours of exposure to oxygen would be greater than that found after days of exposure. Results obtained were consistent with the hypothesis. The 4 to 8-hour response of wound fluid PO₂ to 45% O₂ ten days after wounding was 8 mm Hg. If the

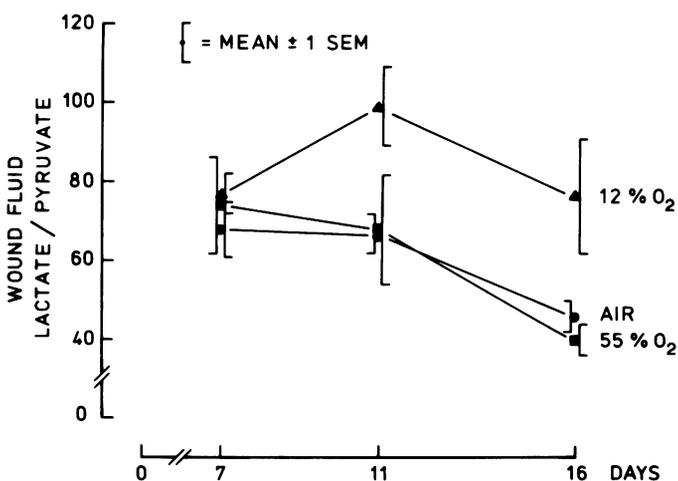


FIG. 6. Effect of changes in inspired oxygen tension on wound fluid lactate/pyruvate ratio.

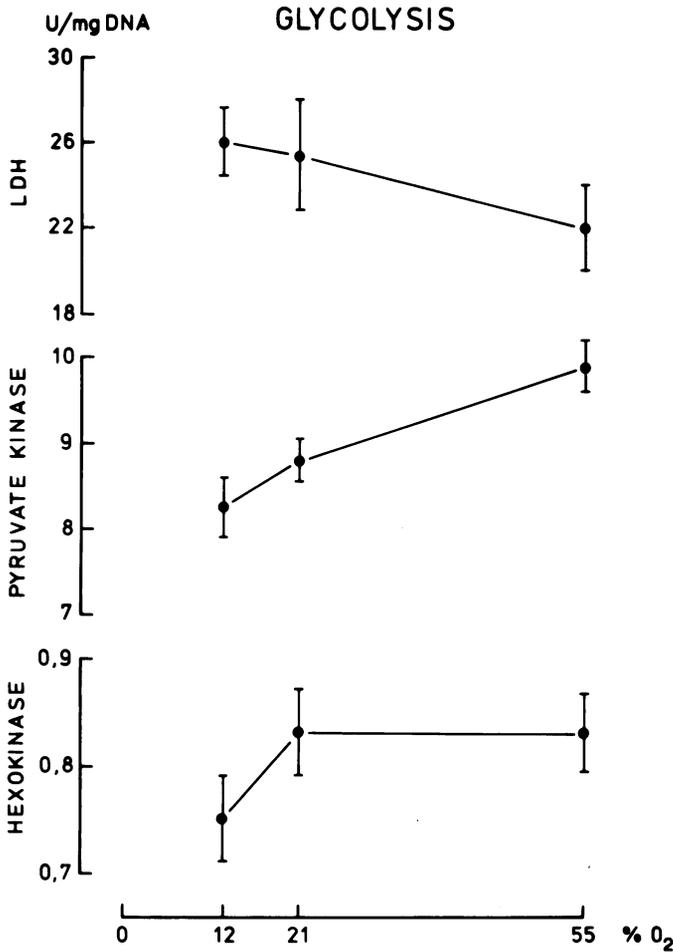


FIG. 8. Effect of changes in inspired oxygen tension on the activities of hexokinase, pyruvate kinase and lactate dehydrogenase in experimental granulation tissue. The determinations were carried out 17 days postimplantation.

exposure was continued for 10 to 15 days, the arterial PO₂ remained high but the wound increment from controls was only 3 mm Hg.⁵ Studies with cellulose sponge implants agree fully with these findings. Chronic exposure to various oxygen concentrations had only slight influence on the wound PO₂ on the 16th day (Fig. 2). In 55% O₂ the increment was only 2 mm Hg whereas an acute exposure to 50% O₂ increased the PO₂ of cellulose sponge implants by 20 mm Hg.¹³

Wound PCO₂

The measurements of wound fluid carbon dioxide (Fig. 3) give further clarification on the mechanism of the action of oxygen. The increase in wound fluid PCO₂ with hyperoxia could be due to enhanced production of carbon dioxide. Other possible factors for the increased PCO₂ would be oxygen-induced vasoconstriction, hypoventilation and decreased carbon dioxide-carrying capacity of hemoglobin due to hyperoxygenation. Vasoconstriction is small in 55% oxygen.⁵ Arterial blood PCO₂ remains

unchanged or rises slightly with increasing ambient oxygen but the increase is much smaller than that found in the wound.⁵ The Bohr effect does not pertain, since breathing of 55% oxygen does not increase the PO₂ in mixed venous blood.¹³ Therefore, the elevated PCO₂ probably results from increased oxygen utilization and carbon dioxide production by wound tissue. The overall decline of PCO₂ values on the 11th day coincides with the time when cell proliferation is diminishing and the most active collagen synthesis is started.⁹

Effect of Oxygen on Granulation Tissue DNA

Increase in the respiratory oxygen concentration from 12 to 55% O₂ had no significant effect on the amount of DNA in granulation tissue (Fig. 7). This agrees with earlier observations on cellulose sponge implants⁹ and suggests that under conditions studied cell proliferation occurred at even rates. On the other hand, chronic exposure to 70% O₂ is known to decrease the amount of DNA in repair tissue.⁹

Effect of Oxygen on Wound Metabolism

In normal wounds glycolysis dominates during the synthesis of collagen.⁷ In glycolysis no oxygen is consumed

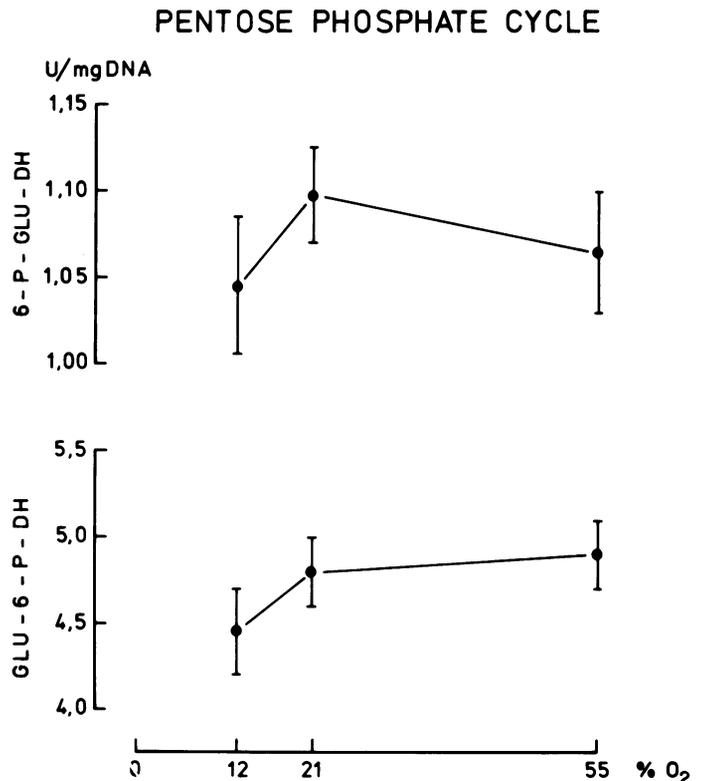


FIG. 9. Effect of changes in inspired oxygen tension on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in experimental granulation tissue. The determinations were carried out 17 days postimplantation.

in the overall process, glucose to lactate. Niinikoski⁹ showed that consumption of glucose in the wound increases with increasing oxygen tension. This agrees with the observations that the activity of hexokinase was decreased in hypoxia and that the activity of pyruvic kinase increased with increasing oxygen tension (Fig. 8).

In hypoxia the amount of wound fluid pyruvate decreased markedly (Fig. 4) probably due to increased reduction to lactate (Fig. 5). Correspondingly, formation of lactate was inhibited in hyperoxia. In most tissues when the oxygen supply is not limiting pyruvic acid is oxidatively decarboxylated to acetyl CoA and carbon dioxide. The measurements of wound fluid PCO₂ (Fig. 3), pyruvate kinase and lactate dehydrogenase (Fig. 8) suggest that this phenomenon also occurs in wounds.

Formation of acetyl CoA from pyruvic acid and coenzyme A is essentially irreversible reaction. Acetyl CoA may be used in a variety of reactions, but its major fate is condensation with oxaloacetate to form citrate, the initial step in oxidation via citric acid cycle. This cycle occurs in mitochondria and provides electrons to the transport system that accomplishes reduction of oxygen while generating ATP. Measurements of the activities of citric acid cycle enzymes suggest that in granulation tissue oxygen supply has a regulatory effect on succinic dehydrogenase, a linking enzyme and important rate modifier between citric acid cycle and electron transfer chain (Fig. 10). This effect is probably indirect and depends on the levels of ADP and substrates. The effect of oxygen on peroxidases was not determined.

Because of limited oxygen supply significant portions of healing wounds probably exist in chronic lack of energy. Hyperoxia shifts the wound metabolism towards aerobic glycolysis. This occurs in parallel with the activation of citric acid cycle. It is possible that this oxygen-induced change in cellular metabolism, i.e. the Pasteur effect, forms one of the basic mechanisms leading to enhanced healing. Other factors may also be involved: increased mean capillary PO₂ increases the amount of oxygen that reaches the cells at the advancing edge of granulation tissue. Therefore, these cells can migrate farther, can retain their synthetic capability farther from the most distal capillary, and some cells can develop an increased synthetic capability allowing faster advancement of vascular supply and faster closure of wound defect.⁵

In addition to oxygen, the supply of substrates is of vital importance for healing. If the Pasteur effect pertains, it indicates that in hyperoxic environment lactic acid is oxidized to carbon dioxide and water, and the energy yield per molecule of glucose is considerably increased. Hence, the energy needs of the cell can be met by consumption of considerably less glucose. In spite of this the granulation tissue consumes increased amounts

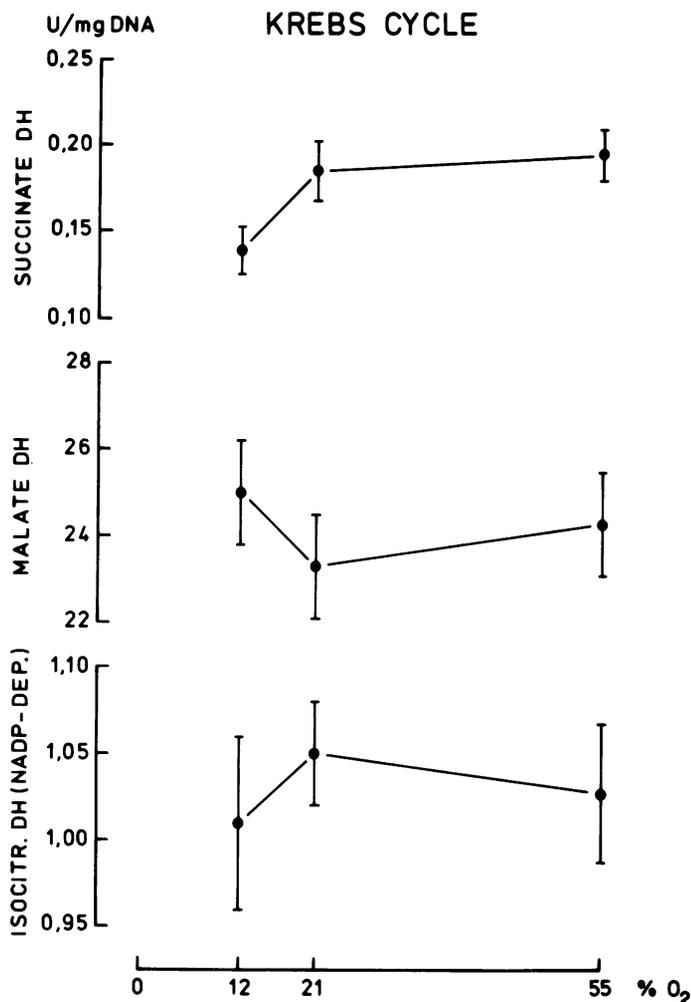


FIG. 10. Effect of changes in inspired oxygen tension on the activities of isocitrate dehydrogenase, malate dehydrogenase and succinate dehydrogenase in experimental granulation tissue. The determinations were carried out 17 days postimplantation.

of glucose if added oxygen is available.⁹ It is possible that cells in the vicinity of the capillary consume glucose so extensively that the supply to the most peripheral cells is limited. This imbalance could probably be corrected by increasing the mean capillary PO₂ which would decrease the glucose utilization of cells adjacent to capillaries. Relatively more glucose would then be available for the most peripheral cells at hypoxic areas. This could explain the elevated glucose consumption in the wound at increased oxygen supply.

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