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Simultaneous Precise Chemoradiation under Inhalation Anesthesia in an Experimental Mouse Tumor Model

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Abstract. *Background: Performing radiobiological experiments under general anesthesia is, in many cases, superior to treatment in unanesthetized animals or with intraperitoneal (i.p.) anesthesia. This is especially true for experiments where highly fractionated treatment schedules are used. Materials and Methods: An anesthesia system was employed to overcome several of the limitations associated with the use of pentobarbital and other i.p.-administered anesthetics in experimental radiotherapy. Results: Several different experiments with a total of 152 mice were performed. The total of all anesthesia exposures amounted to approximately 1,520. The duration of anesthesia ranged from 3 to 5 min per session. No complications related to the anesthetic procedure were observed. Conclusion: The present anesthesia/irradiation setup is a simple, safe and perfectly reproducible system where even multiple fractionated treatments can be performed under anesthesia with excellent tolerance. Our system allows easy and fast handling and immobilization and thus reduces experimental times.*

Clinically relevant radiobiological *in vivo* experiments must reproduce clinical fractionation schedules. This is especially true for experiments where the tumor microenvironment is investigated. In this field, therapy schedules using only single doses or a few radiotherapy and/or chemotherapy fractions are subject to criticism since changes in the microenvironment, which occur during a clinically fractionated therapy and which might be crucial for treatment outcome (15), are neglected by single dose experiments. The therapeutically important effect of reoxygenation (3), for example, is not recognized in single dose experiments, and thus treatment results of those experiments may lead to false conclusions.

In most cases, the use of clinical fractionation schedules means the administration of a high total radiation dose given in many fractions. However, this approach is often problematic. First, fractionated therapies are much more time-consuming, and access to the treatment facilities is limited in clinical departments. Secondly, precision in fractionated radiotherapy is more important than in single dose experiments since every fraction has to be geometrically identically delivered to the right anatomical site. Furthermore, in many experiments radiotherapy is given together with chemotherapy, which might require intravenous infusion procedures or other elaborate handling. Therefore, a reliable and time-saving system for the treatment of experimental tumors, which allows for repeated and precise irradiation together with chemotherapy, is necessary.

In experimental radiotherapy, rodents are often immobilized for local irradiation by use of little lead jigs (5, 8, 9). For this purpose, the animals are urged into small lead containers. The tumor-bearing leg (or back) is stretched out through an opening of the jig, while the rest of the animal is protected against irradiation. This procedure is not optimal for several reasons. The (unanesthetized) animals are able to move in the lead jigs, which means that, in some cases, experimental tumors are not irradiated sufficiently or even not at all. With bigger animals like rats this problem might be even more pronounced. In addition, it is not possible to inject chemotherapeutic agents while the animals are in the jigs.

Other investigators have used intraperitoneal *(i.p.)* injections of anesthetics to immobilize animals (1, 13). However, repeated anesthesia with *i.p.* injections, necessary for multi-fractionated therapy, have many limitations. The depth and duration of anesthesia is hardly controllable and death due to respiratory arrest may occur. Furthermore, physiological parameters such as breathing rate and blood

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oxygen saturation, crucial for radiobiological experiments, cannot be controlled. Moreover, inner organs such as the liver and spleen are quite large in rodents and can easily be injured by *i.p.* injections.

Animal protection has become an important issue in preclinical experiments. This has far-reaching implications, especially for experiments requiring elaborate handling of the animals as is the case for almost all radiobiological and chemotherapeutical experiments. Restraining of awake animals in very small jigs for experimental radiotherapy, for example, is critical from the animal protection point of view, and even legally questionable in many countries. We believe that both experimental and animal protection requirements can optimally be achieved only under inhalation anesthesia. We therefore developed a set-up to simultaneously treat groups of animals with irradiation and chemotherapy injections. This procedure can be performed repeatedly under safe and reproducible anesthesiological conditions.

Materials and Methods

Animals and tumor model. 152 C3H/J mice were supplied by Charles River, Sulzfeld, Germany. At the beginning of the experiments the mice were six months old and weighed on average 27 +/– 3.3 g. All experiments were conducted according to the guidelines and directives set down by the governmental authority, the Tierschutzkommission of Oberbayern, Munich, Germany. The animals were bred and housed under germ-free conditions in the Animal Research Facility of the Klinikum rechts der Isar, Technical University of Munich, Germany. Room temperature in the windowless animal room was 19 to 24˚C. An air conditioning system guaranteed constant relative humidity of 55% and provided complete air exchange three times per hour. Illumination was set at twelve hours per day. The animals were kept in groups of six per cage (Makrolon Type 3 cage). They received standard food for mice and rats (Altromin 1324, Altromin, Lage, Germany) and water without supplements *ad libitum*.

Anesthesia and treatment set-up ('anesthesia/irradiation circuit'). The experimental set-up presented here served as a basic study for further experiments where the toxicity of a variety of combined regimens of chemo-radiotherapy in a mouse tumor model are evaluated. The anesthesia system was designed for a two-week fractionated regime with ten sessions of irradiation plus *i.p.* injections of several chemotherapeutic agents. Anesthesia was needed for every treatment session, *i.e.* each mouse received ten sessions of anesthesia over two weeks.

The anesthesia procedure was initiated in a 900 ml body chamber of acrylic glass (Figure 1), which was preflooded rapidly with pure oxygen and 4% isoflurane. The animals were asleep within one minute. They subsequently received 0.5 ml of saline and were then transferred safely and quickly to the experimental anesthesia/irradiation circuit (Figures 2 and 3). For anesthesia the volatile anesthetic isoflurane (Forene® Abbott GmbH, Wiesbaden, Germany) with an inspiratory concentration of 2% *via* a commercially available anesthesia machine (Sulla 19, Drägerwerk AG, Lübeck, Germany) was used. Pure oxygen was used as the carrier gas.

The experimental set-up ('anesthesia/irradiation circuit') was designed to simultaneously irradiate six animals per session (Figure 3). Anesthesia was maintained with 2% of isoflurane, enough to reach anesthesia stage 3, plane 1 with good relaxation and stable hemodynamic parameters. The depth of anesthesia was proved by reflex testing (pedal withdrawal reflex in the front and the hind leg negative).

Our anesthesia/irradiation circuit consisted of a commonly used breathing tube that we placed in the form of a ring on an acrylic glass plate (Figures 2 and 3). Under the glass plate a heating pad was set to 38˚C to avoid hypothermia during anesthesia. On the inner side of the tube six round openings, each of 18 mm in diameter, were cut out. Six 20-ml syringe cylinders were shortened to a length of 60-mm. For gas exchange, two 10-mm holes were cut into the narrow end of the syringes. These prepared syringes were then connected to the six openings into the narcotic tube (Figure 4). The open end of the syringe was covered with a rubber fingerstall. Each fingerstall was slit so that it served as a nose cone. During anesthesia, the mice's noses were pushed through the slit in the fingerstall into the nose cone (Figure 4). This way the tube could be easily and constantly flooded without leaking isoflurane.

For irradiation a round standard metal tube, 12 cm in diameter, was focussed at the center of the anesthesia/irradiation circuit (Figure 2). Before starting irradiation, the entire tumor-bearing legs were taped toward the center of the circuit, therefore allowing exclusive irradiation of the experimental tumors on the feet and sparing all other structures such as thighs, tails and all inner organs. Immediately after irradiation had been completed (2 min), the chemotherapeutic drugs were injected *i.p.,* while the animals were still under general anesthesia. They were successively removed from their nose cones and were allowed to recover. After approximately 1 minute, they were completely awake and then put back in their cages. The whole procedure lasted between 5 to 7 minutes.

Results

Several different experiments with a total of 152 mice were performed with the set-up presented here. In all experiments, ten repeated sessions of anesthesia within two weeks were performed, so that the total of all anesthesia exposures amounted to approximately 1,520. The duration of anesthesia ranged from 3 to 5 minutes per session. No complications related to the anesthetic procedure were observed. At the end of all anesthesia sessions, the mice, without exception, woke up within 30 seconds. Within another 30 seconds, they started to walk and ate and drank.

In a study investigating cisplatin and tirapazamine toxicity, it has recently been shown that weight loss is an important parameter for the general condition of rodents during radiobiological experiments (submitted for publication by Adam *et al.*, 2004). In the present study, only minimal weight loss was observed with the median minimum weight dropping to 95% of the initial weight (range 86-100%), demonstrating only minimal toxicity. None of the animals in our study died.

Figure 1. *Start of anesthesia in the 900 ml body chamber.*

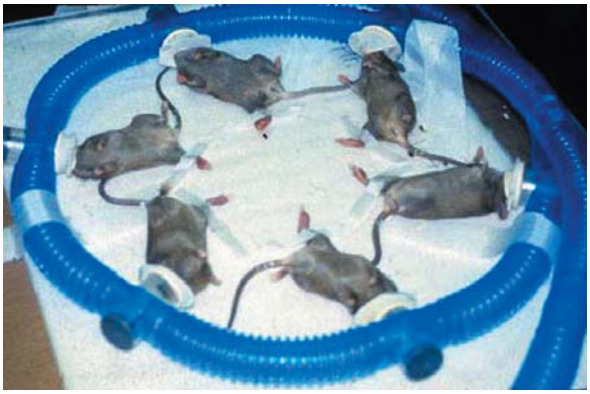

Figure 2. *Anesthesia set-up with animals in serial nose cones.*

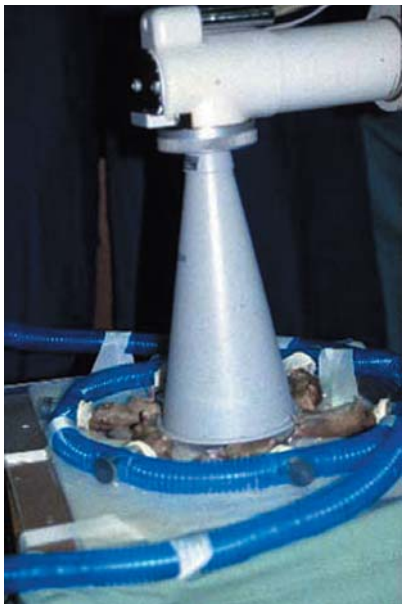

Figure 3. *Irradiation set-up with standard metal tube.*

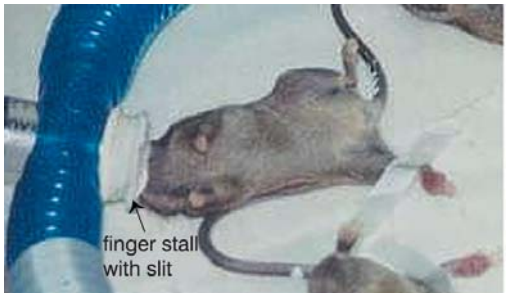

Figure 4. *Animal with nose cone connected to one of the openings into the narcotic tube. During anesthesia the mice's noses were pushed through the slit in the finger stall into the syringe.*

Table I. *Number of animals with histopathological organ changes in relation to the total number of animals evaluated.*

	Number of animals with organ damage/ total number of animals evaluated
Bone marrow	0/12
Kidneys	0/12
Heart	0/12
Stomach	0/12
Liver	3/12
Lung	1/12
Spleen	2/12

For more detailed evaluation of toxicity, all inner organs were histologically examined. To this end, 3-μm slices of a variety of inner organs were stained with Hämalaun-Eosin as described by Romeis *et al.*, 1989. In selected cases, additional examinations were performed using Berliner Blau and/or Kossa staining. No significant damage to the inner organs was observed (Table I).

Discussion

Six to eight weeks is the typical overall treatment time-span of clinical radio- or radio-chemotherapy. During this time, essential changes in the tumor microenvironment occur (10, 20). Tumor oxygenation and reoxygenation, for example, are two tumor characteristics that might determine whether a patient will be cured or suffer from relapse (15). Of course, short-term or single-dose experiments cannot reflect changes that develop in the tumor microenvironment over time, and therefore conclusions for the clinic from those experiments may be misleading. It is therefore necessary

that experimental radiotherapy is performed in a manner as similar as possible to clinically applied regimens. This means that treatment regimens are needed with multiple fractions and prolonged overall treatment time. It is clear that the accuracy, reliability and reproducibility of all procedures are major factors in the practical realization of such experiments. For this reason, we developed a system to treat transplanted experimental tumors under conditions of inhalation anesthesia.

In most animal experiments immobilization procedures are inevitable. Though perfect immobilization can only be done under anesthesia, a lot of investigators perform experiments in unanesthetized animals using small jigs to restrain and shield animals from radiation. The advantage of this approach is that possible pharmacological influences of the narcotics on radio- (or chemo-) sensitivity are avoided. However, jigging of mice without anesthesia can also lead to marked changes in therapy response. This has been proved by Shibamoto *et al.* who were able to show that jigging of unanesthetized animals increased the hypoxic fraction with corresponding radioprotection (19). This is most probably caused by the stress due to jigging, which leads to a shift of physiological parameters (*e.g.* breathing rate and body temperature) which are known to influence radiation sensitivity (25).

Although the influence of inhalation anesthetics on radiosensitivity is an important issue both for experimental and clinical therapies such as intraoperative radiotherapy or fractionated radiotherapy with repeated anesthesia in small children, data on that subject are rare and reveal a confusing picture. Whereas some authors have reported significant radioprotection (6, 18), some reported the opposite (11, 22), while others have described no significant effect (26). Unfortunately, investigators have used all kinds of different immobilization and anesthetic techniques and all kinds of different treatment set-ups, rendering these data very hard to compare. Additional uncertainties in the interpretation of data exist concerning the physical dose distribution for irradiation of small volumes with correspondingly small fields and moving of non-anesthetized animals in their jigs.

Apart from these technical considerations, contradictory responses to irradiation may also have pharmacological causes. Of course, any kind of anesthesia has certain effects on physiological factors, some of which are known to influence radiosensitivity. Changes in body temperature and breathing rate can lead to altered blood supply, blood O_2-saturation and pH, thus leading to changes in tissue oxygenation and altered radiosensitivity (24). The influence on those physiological parameters obviously depends on the depth and duration of anesthesia. Since treatment set-ups and the duration of narcosis vary greatly between the reported studies, this might explain some of the contradictory results. Indeed, Nias and Perry (16), using *i.p.* narcotics and large single irradiation doses, could demonstrate that the radiation response of tumors varies greatly with the time of treatment after the beginning of anesthesia.

To our knowledge, published data on the effects on radiosensitivity is available only for enflurane, a compound very similar to isoflurane, but not for isoflurane itself. It should be pointed out that isoflurane is superior to enflurane for a few reasons (14) and, therefore, only isoflurane is used in human medicine. The data for enflurane concerning modification of radiosensitivity is contradictory. Kal and Gaiser (12) have reported repair inhibition in single dose experiments leading to an increase in radiosensitivity for one rat tumor and foot skin. This is in agreement with the single dose experiments of Thomas *et al.* (23), who also have described a sensitizing effect of enflurane/carbogen compared to only carbogen breathing in mouse lip mucosa. Stüben *et al.* (21) investigated the effects of enflurane on mouse lip mucosa, giving identical radiotherapy with or without anesthesia. Using single dose experiments with large fraction sizes, they also detected a radiosensitizing effect. However, this effect was no longer significant in fractionated radiotherapy, where they found the same isoeffective dose for lower lip desquamation for unanesthetized animals as for those who breathed enflurane/air. Taking into account that *in vivo* radiobiology should investigate the effects of fractionated treatments with fraction sizes adopted from clinical regimes, the possible sensitizing effect of large single doses does not seem to be of major relevance. In our study, anesthesia did not appear to have a major impact on radiosensitivity since tumor doubling times (median 4 days; range 3-5 days) were very similar to data reported in the literature for untreated control animals (4, 9).

In agreement with Ang *et al.* (2), we also found that inhalation anesthesia with pure oxygen as the carrier gas and control of body temperature caused no morbidity, even after multiple treatments. Plummer *et al.* (17) have investigated the effects of a 30-week exposure to enflurane in rats. Even after such a prolonged exposure, anesthesia was very well tolerated. No hepatocellular necrosis, increase in serum alanine aminotransferase activity or in liver size was observed after exposure to enflurane. No organ damage or weight loss was observed in our animals after ten anesthesia sessions each. Our experimental procedure with multiple settings of anesthesia appears to be safe and causes no additional toxicity.

A major advantage of inhalation anesthesia compared to *i.p.* injections of anesthetics is that the depth and duration of anesthesia can be controlled optimally with inhalation anesthesia. *I.p.* injections produce longer induction times and can cause deaths due to unpredictably long recovery times. A

single *i.p.* injection results in deep surgical anesthesia for 1-2 hours and a hangover period of some hours. Respiratory arrest may occur, especially when the *i.p.* application is repeated a number of times within a short period of time (2). Furthermore, several studies have indicated that pentobarbital anesthesia may decrease radiosensitivity. This is probably caused by induction of hypoxia (7) and by the long duration of anesthesia. Full recovery after pentobarbital anesthesia can take up to one hour (7). In contrast, both the induction time and time to full recovery is very short and can easily and precisely be controlled with inhalation anesthesia. Moreover, physiological parameters such as breathing rate and temperature can easily be regulated by varying the depth of inhalation anesthesia and by constantly measuring and regulating the body temperature with probes and heating pads, respectively. It should also be mentioned that the rapid awakening of the animal diminishes toxicity by minimizing disturbance of food ingestion. This is crucial since mice have a high metabolic rate and must therefore quickly regain normal conditions.

Conclusion

Performing radiobiological (and chemotherapeutical) experiments under general anesthesia is, in many cases, superior to treatment in awake animals and *i.p.* anesthesia. This is true for technical, pharmacological and also animal protection considerations. Our anesthesia/irradiation circuit is a simple, safe and perfectly reproducible system, where even multiple fractionated treatments can be performed under anesthesia with excellent tolerance. Our system allows easy and fast handling and immobilization and thus a timesaving realization of experiments. The versatility of the system is high: the kind of anesthetics or mixtures of breathing gases to be used, the possibility to irradiate under ambient, hypoxic or clamped conditions and, with few alterations, the number of animals to be treated can be changed if required. Furthermore, the system can be connected to a mobile unit. This is important when no radiotherapy unit is available exclusively for experimental purposes.

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