Effects of dimethylthiourea, melatonin, and hyperbaric oxygen therapy on the survival of reimplanted rabbit auricular composite grafts

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Microvascular reconstruction currently offers the best chance of survival for an ear segment reimplanted after amputation. Unfortunately, this technique is possible only when ear and scalp vessels remain intact. Direct reattachment of the amputated segment as a composite graft is less reliable because survival is poor for grafts larger than 2 cm in diameter. However, if survival could be improved, direct reattachment would be an attractive alternative in situations in which microsurgical revascularization is not possible. Certain pharmacologic agents have been shown to enhance the survival of composite grafts. This study demonstrated that hyperbaric oxygen, dimethylthiourea, and melatonin significantly affected the survival of reimplanted auricular composite grafts at day 7. However, by day 21 the average percentage of survival for all groups approached 13% to 14%. Dimethylthiourea had the most beneficial effect on survival early in the postoperative period, whereas the hyperbaric oxygen group demonstrated the poorest survival. (Otolaryngol Head Neck Surg 1999;121:231-7.)

Traditional methods of treating an avulsed or amputated ear have included simple reattachment of the ear as a composite graft,[1-5] dermabrasion of the auricular epithelium followed by burial in a mastoid pocket for later retrieval,[6-9] and creation of a variety of local and distant flaps to salvage the ear.[10-14] Although none of these techniques has been entirely satisfactory, microsurgical techniques have yielded impressive results. However, unless the ischemic interval is short and the amputated ear and the adjacent scalp vessels remain intact, the chance of successful microsurgical replantation is low.[10] Furthermore, the operation is lengthy and requires considerable surgical skill, specialized training, and costly equipment.[11] In situations in which microsurgical replantation is not feasible, simple direct reattachment of the amputated ear would be a valuable option if survival of the graft could be improved. Auricular composite grafts are commonly used for reconstruction of various sites in the head and neck, including the alar rim, columella, lateral nasal wall, ear, lip, and trachea.[15]

Unfortunately, reconstructions using composite grafts are currently limited by the size of the grafted tissue. Converse[16] reported that the most successful grafts were smaller than 2 cm in diameter and that, ideally, no part of the graft should be farther than 1 cm from the free margin. Although successful cases of subtotal and total ear reattachment have been reported in the literature,[1-5] graft survival has generally been poor.

A review of the literature reveals that the survival of composite grafts can be enhanced by certain pharmacologic agents.[17-20] These studies demonstrate that methylprednisolone sodium, dimethylsulfoxide, chlorpromazine, and indomethacin significantly improve survival of reimplanted auricular cartilage grafts in rabbits. Methylprednisolone seems to be the most effective agent studied to date.[17-20] However, investigation of other agents is required before direct reimplantation can be considered a satisfactory alternative to microvascular surgery in the reconstruction of the amputated ear.

This randomized, double-blind, placebo-controlled, prospective animal study examines the effects of dimethylthiourea (DMTU), melatonin, and hyperbaric oxygen (HBO) therapy on the survival of reimplanted auricular cartilage grafts after amputation. DMTU, an oxygen free radical scavenger, and HBO both improve the survival of tissue flaps in animals and human beings.[21-26] Similarly, melatonin acts as a potent oxygen radical scavenger and protects tissues against oxidative stress in a variety of animal models.[27-31]

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Fig 1. Rabbit auricular composite graft immediately after reimplantation.

METHODS AND MATERIAL

Under the approval of the Laboratory Animal Care and Use Committee of Naval Medical Center Portsmouth and in accordance with the Animal Welfare Act, 44 female New Zealand white rabbits weighing 3 to 4 kg were anesthetized with ketamine 40 mg/kg and xylazine 5 mg/kg administered by intramuscular (IM) injection in the anterior thigh quadriceps muscle group. Each ear tip was shaved, and a 1% solution of lidocaine was injected at the base of each ear to provide additional anesthesia. A line was drawn 2 cm from the distal tip of each ear perpendicular to the long axis. The surgical field was then prepared with Nolvasan scrub and draped in a sterile fashion. All animals received 84,000 U/kg of subcutaneous penicillin G benzathine and penicillin G procaine before the operation. A scalpel was used to amputate the distal 2 cm of each ear along the line previously drawn. This amputated portion was then immediately reimplanted with interrupted 6-0 Prolene sutures to approximate the skin and perichondrium on the ventral and dorsal surfaces (Fig 1). Bacitracin ointment was gently applied to the suture line at the end of each procedure. The time of surgical procedure, as well as the order of ear reattachment (right vs left), was recorded. Dressings, splints, or collars were not required because the animals did not scratch the grafts.[17-20]

The animals were then randomly assigned to 1 of the 4 experimental groups: (1) no treatment, (2) HBO treatment, (3) DMTU treatment, and (4) melatonin treatment. From this point, when handling and examining the rabbits, the investigators were blinded as to which treatment each rabbit had received. The method by which the rabbits were tracked throughout the study was determined by the animal laboratory staff.

Animals receiving HBO treatments were placed in a 25-ft^3 hyperbaric recompression chamber and subjected to a pressurized oxygen infusion that elevated atmospheric pressure from 1 ATA (atmosphere absolute) to 2.5 ATA in 10 minutes. The animals were maintained at this constant pressure for 90 minutes and then slowly decompressed in 10 minutes.[23-26] The HBO treatments were started within 4 hours of reimplantation and were administered once a day at the same time for the next 5 days. No anesthesia was required for these treatments because of the slow rate of compression and decompression.

Animals receiving DMTU were given a 500-mg/kg dose in 5 mL of normal saline solution by intraperitoneal injection within 5 minutes after reimplantation of the amputated ears and then daily for the next 5 days. Animals receiving melatonin were given a 500-mg/kg dose in 5 mL of normal saline solution by intraperitoneal injection within 5 minutes after reimplantation of the amputated ears and then 5 mL of normal saline solution daily for the next 5 days. Control animals and those receiving only HBO were given 5 mL of normal saline solution intraperitoneally within 5 minutes after reimplantation and then daily for the same period.

Ear viability was assessed immediately after surgery and at 1, 2, and 3 weeks after surgery. The percentage of survival was determined with Folex Film Systems PPC Transparencies (x-300 clear) as templates traced from the viable portion and total area of each reimplanted graft. A percentage of survival was then calculated as a ratio of template weights (%Survival = Weight of template of viable portion of graft ÷ Weight of template of entire graft). Furthermore, an additional method used to determine the percentage of survival involved standardized photodocumentation of the ears with a metric ruler in the background as a reference. Photographs were scanned with an Epson ES 1200C flatbed scanner and Corel Photopaint for Windows 7.0 software. A Nikon F3 with a Nikkor 120-mm medical lens and SB-21 ring-flash mounted on a photography copy stand was used to photograph each ear at a fixed reproduction ratio of 1:3. UTHSCSA Image Tool for Windows 1.28 software was then used to analyze the scanned images and determine the percentage of survival. The animals were then euthanized by the laboratory staff with pentobarbital 100 mg/kg intravenously.

Data were analyzed with the Kruskal-Wallis test, a non-

Table 1. Mean percentage of survival at days 7, 14, and 21 determined by template weight

Group	Day 7	Day 14	Day 21	F *P* value
Control	0.7218	0.1629	0.1228	0.001
HBO	0.0000	0.1065	0.1938	<0.001
DMTU	0.9160	0.4433	0.0036	0.001
Melatonin	0.8239	0.1449	0.1766	0.001
Mean	0.5728	0.1940	0.1341	—
K-W *P* value	<0.001	0.008	0.007	—

K-W, Kruskal-Wallis test; *F,* Friedman test.

Table 2. Mean percentage of survival at days 7, 14, and 21 determined by photographic analysis

Group	Day 7	Day 14	Day 21	F *P* value
Control	0.2320	0.0661	0.1079	0.060
HBO	0.0094	0.0786	0.1538	0.001
DMTU	0.5399	0.1743	0.1471	0.004
Melatonin	0.1815	0.0840	0.1355	0.121
Mean	0.2123	0.0939	0.1350	—
K-W *P* value	<0.001	0.337	0.894	—

K-W, Kruskal-Wallis test; *F,* Friedman test.

parametric analysis, to determine whether the administration of DMTU, melatonin, or HBO significantly influenced the average survival area of reimplanted ears in New Zealand white rabbits at 1, 2, and 3 weeks. Friedman testing was used to determine the effects of each treatment on the survival of reimplanted ears during the 3-week period. Finally, results obtained by use of the ratio of template weights were compared with those determined by photographic analysis.

RESULTS

The mean percentage of survival for the control, HBO, DMTU, and melatonin groups at days 7, 14, and 21, determined by the ratio of template weights, is presented in Table 1. The mean percentage of graft survival for each group at days 7, 14, and 21, determined by photographic analysis, is presented in Table 2. At day 7, both methods demonstrated that the DMTU group had the greatest percentage of graft survival (91.60% by template weight and 53.99% by photographic analysis), whereas the HBO group had the poorest graft survival (0.00% by template weight and 0.94% by photographic analysis). At day 14, both methods of measurement still demonstrated the greatest survival in the DMTU group (44.33% by template weight and 17.43% by photographic analysis), but the relationship of percentage of survival among the other groups differed by method. At day 21, both methods measured the best survival in the HBO group (19.38% by template weight and 15.38% by photographic analysis).

The Kruskal-Wallis test was used to determine whether treatment with HBO, DMTU, or melatonin affected graft survival at day 7, 14, or 21. Template weights showed a significant difference in graft survival among the 4 groups at days 7 ($P < 0.001$), 14 ($P = 0.008$), and 21 ($P = 0.007$). However, photographic analysis found a significant difference in graft survival among the 4 groups only at day 7 ($P < 0.001$). At days 14 and 21, the photographic method of determining graft survival did not find a significant difference among the 4 groups ($P = 0.337$ at day 14, $P = 0.894$ at day 21).

Friedman testing was used to determine the effects of each treatment on the survival of reimplanted ears within each group during the 3-week period. This analysis revealed that the change in survival within the control and melatonin groups during the 3-week study was not statistically significant when measured by photographic analysis ($P = 0.060$ and 0.121, respectively).

Five rabbits developed extreme lethargy and poor oral intake during the course of this study, which resulted in dehydration requiring intravenous fluid resuscitation. These animals did not resume adequate oral intake and required euthanasia before the completion of this study. Three of these animals had received DMTU, and 2 of these animals had received melatonin.

DISCUSSION

The rabbit has been reported to be an excellent model to study the survival of composite grafts and is currently the most accepted animal in which to study auricular amputation.[17-20,32] Studies report the survival of untreated reimplanted rabbit ears to be approximately 1% to 13% at 3 weeks.[18,20] This study examined the individual effects of DMTU, melatonin, and HBO therapy on the survival of the reimplanted ear in the New Zealand white rabbit.

A review of the literature revealed that the survival of composite grafts can be enhanced by certain pharmacologic agents. Hartman and Goode[17] reported that rabbits receiving 30 mg/kg IM methylprednisolone sodium succinate daily during the first 4 postoperative days after reimplantation of an auricular composite graft showed a significant increase in graft survival compared with controls (75.4% compared with 41.0%). Aden and Biel[18] confirmed that 30 mg/kg IM methylprednisolone administered to rabbits 1 hour before surgery and continued for 7 days after surgery produced significant less graft necrosis than in controls (28.75% compared with

76.69%). In the same study, preoperative dimethylsulfoxide (40 mg/kg IM), chlorpromazine (15 mg/kg IM), and indomethacin (3 mg/kg IM) also produced significant decreases in graft necrosis, but to a lesser extent. These studies used a rabbit model in which a 2-cm-diameter circular full-thickness auricular composite graft was harvested, rotated 180 degrees, and then reattached.

Henrich et al[20] also used the rabbit to create a model for auricular amputation by incising the distal 2 cm of the rabbit ear as a composite graft and then simply reattaching it. Treatment with 30 mg/kg IM methylprednisolone immediately after surgery and continued for 5 days and treatment with 30 mg/kg IM methylprednisolone after storage in iced saline solution for 90 minutes showed a significant increase in graft survival compared with control treatment (23.2% and 15.5%, respectively, compared with 1.3%). Therefore certain pharmacologic agents have been shown to improve survival of composite grafts in the rabbit model.

DMTU, an oxygen free radical scavenger, has also been shown to increase the survival of skin flaps in animals.[21,22] Presumably, DMTU acts by selectively scavenging the hydoxyl radical, one of the most potent oxidants produced in postischemic tissue.[21] Although prolonged ischemia alone ultimately produces tissue death, much of the damage occurs during reperfusion of ischemic tissues when molecular oxygen is again available.[33-35] This phenomenon can be explained by the conversion of xanthine dehydrogenase, an enzyme widely present in tissues, to xanthine oxidase during periods of hypoxia, which are present during ischemia. During reperfusion, xanthine oxidase uses molecular oxygen to reduce water and produce superoxide. Superoxide is a free radical, a molecule containing an odd number of electrons, which makes it extremely reactive. It is capable of participating in long chain reactions that generate even more free radicals, such as hydroxyl. Free radicals most likely result in cell death through the peroxidation of unsaturated fatty acids present in cell membranes.[25] Specifically, fatty acids are dehydrogenated by free radical attack to give rise to peroxide radicals. The peroxide radicals further dehydrogenate fatty acids to produce hydroperoxide, which participates in the propagation of peroxidation and causes the deterioration of cell membranes—ultimately causing cell death. Thus free radical scavengers have been shown in many studies to increase survival of skin flaps in animals.[36-41] Administration of DMTU may prove effective in increasing survival of the reattached ear in the rabbit model.

Melatonin, a neurohormone produced by the pineal gland, has also recently been discovered to act as a free radical scavenger. In fact, melatonin has been categorized as the most potent physiologic scavenger of hydroxyl radicals identified to date.[27,28] Although found in all tissues, melatonin is believed to function as a highly important antioxidant in the brain.[29] Pierrefiche et al[30] demonstrated that melatonin administered to mice causes a dose-dependent decrease in elevated levels of plasma glucose resulting from pancreatic toxicity caused by administration of alloxan. This substance results in the selective oxidative destruction of insulin-secreting β cells of the islets of Langerhans. Melatonin is also shown to prevent experimental membrane lipoperoxidation, which is thought to be the mechanism by which free radicals cause cell death.[30,31] Finally, melatonin inhibits cataract formation in newborn rats given buthionine sulfoximine, a glutathione synthesis inhibitor.[42] Glutathione, an endogenously produced antioxidant, prevents oxidative damage that is a causative factor in cataract formation. Melatonin's antioxidative abilities may also enhance the survival of the reattached ear in the rabbit model.

HBO has similarly been shown to improve survival of tissue flaps and grafts in animals and human beings. Jurell and Kaijser[23] found that rats with pedicled flaps treated with HBO had approximately twice the survival area of controls. Manson et al[24] showed that animals treated with HBO demonstrated 3 times more distal growth of capillaries than did controls. Similarly, Nemiroff and Lungu[25] found that the number and size of blood vessels in the microvasculature of skin flaps are significantly greater in animals treated with HBO than in controls. In a clinical study, Perrins and Cantab[26] demonstrated complete survival of grafts in 64% of HBO-treated patients, as opposed to only 17% of controls. Finally, Zhao et al[43] reported that HBO delivered at 2 ATA for 90 minutes daily for 5 days increased the amount of granulation tissue formation in ischemic rabbit ear ulcers. By increasing the amount of oxygen dissolved in the plasma and tissues, HBO has been shown to block lipid peroxidation, preserve intracellular adenosine triphosphate, and promote neovascularization.[44] By these mechanisms, HBO should also have a positive impact on the survival of reimplanted ears in the rabbit model.

In this study the distribution characteristics of the data did not lend themselves to parametric testing. Specifically, the template method measured 0% survival in every reimplanted ear in the HBO group on day 7. Because of this absence of variance in the HBO group, the data failed to meet the assumptions of parametric testing.[45] Therefore a nonparametric analysis, such as the Kruskal-Wallis test, was required to determine whether treatment with HBO, DMTU, or melatonin affected graft survival at days 7, 14, and 21.

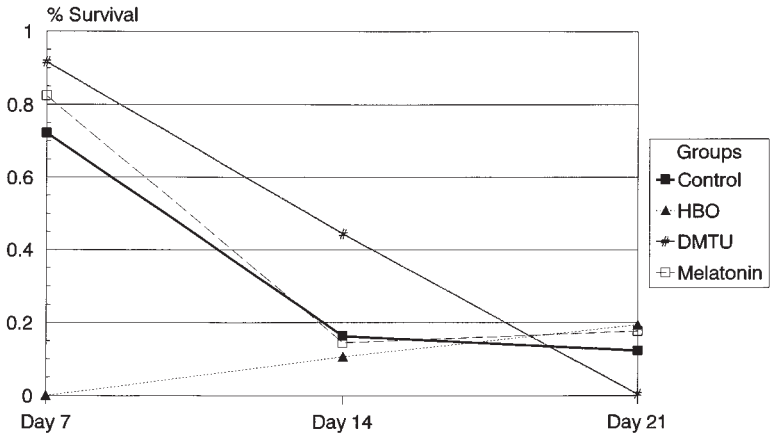

Fig 2. Average percentage of survival for each group by template weights.

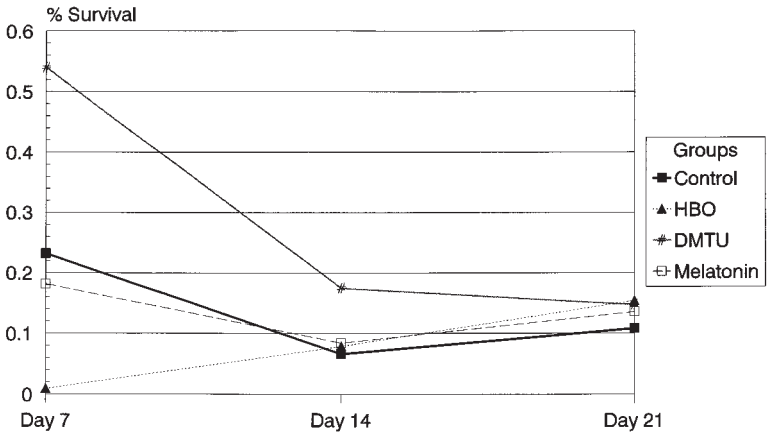

Fig 3. Average percentage of survival for each group by photographic analysis.

At day 7, a large difference in percentage of survival was observed among the 4 groups when both the ratio of template weights and photographic analysis were used; this difference gradually decreased over time. At day 21, a significant difference in graft survival among the 4 groups was only noted when determined by template weights, and not by photographic analysis. The mean percentages of graft survival of all groups at day 21 determined by template weights and photographic analysis were 13.41% and 13.50%, respectively. These results agree with those of published reports of graft survival in untreated control groups of 1% to 13%.[18,20] Therefore HBO, DMTU, and melatonin did have a significant effect on graft survival at day 7, which was no longer evident by photographic analysis at day 21. It would be interesting to examine the effects of extending the course of these therapies on survival after day 21.

The Friedman test, another nonparametric analysis, was used to determine whether these treatments affected survival within each group during the 3-week period. When measured by template weights, all 4 groups showed a significant change in survival during the 3-week period (Fig 2). However, photographic analysis did not reveal a significant change in survival in the control and melatonin groups by the end of 3 weeks (Fig 3).

The 2 methods used to determine graft survival in this study have been well described in the literature.[17-19,21,22,32,40,41] However, this is the first study to directly compare these 2 methods when measuring graft survival. At day 7, both methods demonstrated that the DMTU group had the greatest percentage of graft survival. Because free radicals are produced mainly during the reperfusion of reimplanted grafts, it is not surprising that the improved survival of the DMTU group was seen early in the postoperative period. The decline in survival of the DMTU group noted at 3 weeks was believed to be most likely the result of venous conges-

tion of the reimplanted grafts. This was consistent with the edematous, ecchymotic, and purple appearance of the grafts at 2 weeks.

Both methods of analysis also showed that the HBO group had the poorest graft survival at day 7. However, determination by template weights demonstrated that the melatonin group had a better percentage of survival than the control group (82.39% vs 72.18%), whereas photographic analysis revealed that the control group had a better percentage of survival than the melatonin group (23.20% vs 18.15%) at day 7. Furthermore, survival was noted to improve from days 14 to 21 in the control, HBO, and melatonin groups. Finally, the use of templates tended to overestimate graft survival in each group at days 7 and 14 when compared with the results obtained by photographic analysis.

These discrepancies seem to bring one or both methods of measurement into question. However, they reflect the fact that accurate measurement was complicated by ecchymosis and edema caused by venous congestion early in the postoperative period. By day 21, resolution of congestion and clear demarcation between necrotic and viable tissue made analysis straightforward. Also, the data showed increasing survival in several groups during the 3-week period. This overprediction of tissue loss during the first 2 weeks is again attributable to venous congestion and to our practice of considering dusky portions of the grafts to be nonviable for purposes of measurement. In fact, this pattern of improving survival over time is described in studies using similar methods.[32] At day 21, however, the mean percentage of graft survival determined by both methods approached similar results.

We found significant morbidity in the DMTU and melatonin groups. The doses and routes of administration of DMTU and melatonin in this study were based on those used in previously published rabbit, rat, and mice experiments.[21,22,46-50] Before the completion of this study, 3 animals in the DMTU group and 2 animals in the melatonin group had to be euthanized because of cessation of oral intake and severe dehydration. These animals, as well as randomly chosen animals from all 4 groups that survived the full length of the study, underwent gross and histologic examination during autopsy at the Naval Medical Research Institute, Bethesda, MD. The animals in the DMTU group demonstrated mostly normal gross findings and severe diffuse pulmonary edema and renal congestion on histological examination. These findings were believed to be caused by terminal cardiovascular collapse and not by any direct toxic effect of DMTU. No significant differences were found between those animals euthanized early and those surviving the entire length of the study in the DMTU group. However, 1 animal euthanized early in the DMTU group displayed multifocal mild mucosal hemorrhage in the stomach. No apparent cause for the mucosal hemorrhage could be found. There are no reports of DMTU toxicity in the literature.[21,22]

In the melatonin group, some of the animals demonstrated acute hepatic inflammation. One animal who was euthanized early on demonstrated diffuse severe hepatic necrosis consistent with acute toxic hepatic necrosis. This finding is significant because there are no reports of any toxic effects caused by melatonin administration in the published literature.[27-31,42] Furthermore, it has been reported that melatonin has no known median lethal dose and is nearly nontoxic.[51] The production and sale of melatonin is currently unregulated, and it is widely available to the public without prescription. Our experience seems to indicate that melatonin may have toxic effects on the liver in rabbits when given by intraperitoneal injection, and further study into the toxicity of melatonin is warranted.

CONCLUSION

Direct reattachment of an amputated ear segment would be an attractive alternative in situations in which microsurgical revascularization is not feasible if survival could be improved. This prospective, double-blind, placebo-controlled animal study demonstrated that HBO, DMTU, and melatonin significantly affected survival of reimplanted auricular composite grafts at day 7, but this effect was not present by day 21. The DMTU group demonstrated the best percentage of survival at day 7, whereas the HBO group demonstrated the poorest survival. The beneficial effect of free radical scavengers was evident in the early postoperative period. Furthermore, it appears that venous congestion may have played a role in the tissue death observed by 3 weeks. A study designed to examine the effects of lengthening the course of drug therapy on long-term survival of reimplanted composite grafts, possibly with the addition of leech therapy, is indicated. However, the autopsy findings in the DMTU and melatonin groups may indicate an unacceptable level of drug toxicity at the doses used in this study, which would make administration of these agents impractical even if survival were improved.

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