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Preventing Testicular Damage in Genital Burns With Cooling: An Experimental Study

Genital Yanıklara Bağlı Testis Hasarlanmasına Soğutma Tedavisinin Etkileri: Deneysel Bir Çalışma

Ayşe Ebru Abalı[1], Handan Özdemir[2]

[1] Başkent Üniversitesi Yanık ve Yangın Afetleri Enstitüsü Ankara Yanık Ünitesi
[2] Başkent Üniversitesi Tıp Fakültesi, Patoloji Anabilim Dalı

Aim: Investigate changes in testes after cooling scrotal burns with melaleuca hydrogel after burn trauma.

Materials and Methods: Twenty male Sprague-Dawley rats were divided into 4 groups of 10 testes each. Five animals in the sham group were anesthetized; both testes were removed through a lower transverse abdominal incision. In the burn-induced groups, a tin container filled with boiling water was used to induce burns. In group T30, scrota were fixed in a hole in a 25×30 cm porcelain wall tile plate and contacted the steam for 30 seconds; in groups T60 and TB60 (melaleuca hydrogel application group), the same procedure was done for 60 seconds. Excisional biopsies of the testes in all groups were done 1 hour later. Proliferation indexes for spermatogenic cells were measured using a PCNA labeling index. Apoptotic activity in spermatogenic cells was measured using the TUNEL index. Epidermal and dermal damage to the skin was measured semiquantitatively.

Results: No significant differences were found regarding proliferative indexes in any group. Increased apoptotic activity was observed in the T30 group, testicular degeneration began with a high apoptotic rate in spermatogenic cells at 60 seconds (P<.05). Application of melaleuca hydrogel to the scrotum after 60-second steam-burn trauma decreased apoptotic activity levels to those of the T30 group (immune-histochemistry) (P>.05). No degeneration of the spermatogenic cells was seen in the TB60 group.

Conclusions: Immediate cooling by applying melaleuca hydrogel after burn trauma decreases the amount of testicular damage in a rat model of experimental scrotal steam burns.

Key Words: ***Burns, Genital, Cooling, Testis, Experimental, Melaleuca hydrogel***

Amaç: Bu çalışmanın amacı yanık sonrası akut dönemde soğutma ve analjezi amacıyla kullanılan malelauca hidrogel uygulamasının genital bölge yanıklarında testis hasarlanması üzerine etkilerini araştırmaktır.

Gereç ve Yöntem: Yirmi adet erkek spraque-dawley sıçan (250 ± 20gr) 4 gruba ayrıldı. 5 denekten oluşan ' 'sham' grubu'nda anestezi altında alçak transvers karın kesisi ile girilerek her iki testis gövde dışına alındı. Bir saat bekletildi. Yanık grupları için kaynar su içeren bir kap kullanıldı. Her bir denek anestezi altında fayans bir plaka üzerine yerleştirildi, fayans plaka üzerinde oluşturulmuş olan deliğin içine her iki testis skrotumile birlikte sabitlendi. T30 grubu kaynar su buharına 30 saniye, T60 grubu kaynar su buharına 60 saniye maruz bırakıldı. Yanık sonrası malelauca hidrojel uygulanan TB60 grubu ise 60 saniye süreyle buharla temas ettirildi. İşlemlerden bir saat sonra testisler eksize edildi. Biyopsi materyali immune-histokimyasal, histolojik çalışmalar için hazırlandı. Testislerdeki apoptotik aktivite için TUNEL indeksi, scrotal derideki epidermal ve dermal hasarlanma için semi-kantitatif yöntem kullanıldı.

Bulgular: Sham grubu ile karşılaştırıldığında T30 grubunda spermatogenik seri, Leydig ve Sertoli hücrelerinde artmış apoptotic aktivite saptandı(p<0.05). T60 grubunda apopitoz artışına ek olarak testis dokusunda dejenerasyon bulguları görüldü. TB60 grubunda ise apopitoz düzeyinin T30 grubundakiyle eş düzeyde olduğu ve dejenerasyona rastlanmadığı gözlemlendi (p>0.05).

Sonuç: Sonuçlarımıza göre, deneysel buhar yanığı modeliyle oluşturulan skrotal yanık sonrası, akut dönemde malelauca hidrojel uygulaması yoluyla soğutmanın, testis dokusundaki hasarlanmayı azaltmaktadır.

Anahtar Sözcükler: ***Yanık, Genital, Soğutma, Testis, Malaleuca hidrojel***

Cooling the burn wound has been used empirically for centuries to reduce pain and decrease mortality (1). Repeat cooling of the burn wound with water-soaked gauze or hydrogel has been shown to reduce the surface temperature as well as the state of dehydration of a burned zone. This treatment also has been found to reduce pain and damage due to perilesional vasodilatation (2,3).

Melaleuca hydrogel is a hydrogel dressing composed of water (96%) and melaleuca (1.03%). This hydrogel promotes hydration in the burn zone, while the essential oil of the tea tree (melaleuca) prevents infection via bacteriostatic action (4, 5).

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Corresponding Author

Dr. A.Ebru Abalı
GSM: 0 542 723 00 31
E-mail: aesakallio@gmail.com
Baskent University Hospital of 5th Street Polyclinic , 53. Sok. NO: 48 , Bahçelievler- Ankara

Scrotal thermal injury, occurring frequently in major burns, receives less attention than the other parts of the injured body (6,7). However, past cytologic and histologic research on the testes have shown that even mild heat stress induces some changes in the structure of testicular tissue (8-10). In addition, severe burn injury to the scrotum induces damage to the testes as well as to the scrotal skin. The first phase of our experimental studies on testicular thermal trauma showed that severe thermal injury has an immediate negative effect on spermatogenic cells and Sertoli and Leydig cell populations in experimental conditions (11).

The aim of this study was to investigate the therapeutic effects on the scrotal skin and testicular tissue of applying melaleuca hydrogel to the scrotum immediately after severe thermal injury.

MATERIALS AND METHODS

Twenty male Sprague-Dawley rats weighing 250 ± 20 g were used. The animals were supplied by the Baskent University Laboratory Animal Breeding Center in Ankara, Turkey. The study was conducted at the Baskent University Experimental Research Center. Animals were housed in environments that had been standardized for light and temperature and were given access to standard rat chow and water ad libitum. All animals received humane care in compliance with the European Convention on Animal Care. The study protocol was approved by the Ethics Committee for Experimental Research on Laboratory Animals at Baskent University.

Rats were randomly divided into 4 groups of 5 animals each (10 testes per group). Each testicle was considered an individual experimental entity. The sham group (S group) consisted of 10 healthy testes. In this group, rats were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Alfamine 10%, Alfasan, Holland) and 10 mg/kg xylazine hydrochloride (Rompun 2%, Bayer, Turkey). Both testes were removed through a lower abdominal transverse incision. Eight testes were prepared for histologic examination under light microscopy, 2 testes were divided in half, and 1 half was used for ultrastructural examination under transmission electron microscopy, while the other halves were prepared for light microscope and immunohistochemical investigations

Burn model

Before designing the burn model, a preliminary study was done on 4 male Sprague-Dawley rats. This preliminary study sought to determine the exact contact areas that would create partial-thickness and full-thickness burns on the scrotal skin. A 15 × 25 × 25 cm^3 tin container was filled with distilled water until it was one-third full. The container was heated on an electric heater until the water began to boil (90°C) with a high amount of steam. The heater was then kept at a constant temperature of 90°C, and boiling water was added to the container to maintain a constant liquid level. Rats were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. Each animal was placed on a 25 × 30 cm porcelain wall tile plate with a 2-cm hole one-third of the way up, centered between the 2 long sides of the plate. The animals' scrotum, with both testes, was put into this hole. The animals' extremities were fixed with plaster onto the porcelain wall tile plate. The porcelain wall tile plate with the rat fixed on its outer surface was placed into the container of boiling water. Rats were kept in the boiling water for 30 seconds for superficial partial-thickness burns; 45 seconds caused no significant changes in dermal damage; however, 60 seconds created deep partial-thickness burns, and 90 seconds resulted in full-thickness burns. These results suggested mean times for severe thermal injury to the testes. The longer the skin contacted the steam, the more coagulation necrosis was observed. The protocol for the present study was based on the results of this preliminary study.

A 15 × 25 × 25 cm^3 tin container was filled with distilled water until it was one-third full. The container was heated on an electric heater until the water began to boil at 90°C and produce a high amount of steam. The heater was then kept at a temperature constant of 90°C, and boiling water was added to the container to maintain a stable liquid level. Rats were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. Each animal was placed on the porcelain wall tile plate in the same way explained above. For the T30 group, contact between the scrotum and the steam lasted 30 seconds. This procedure was the same for the other 2 groups , only there were 60 seconds of contact for the T60 group (11). For the present study Melaleuca hydrogel (Burnshield, Johannesburg, South Africa) was applied to the scrotum immediately after a contact period of 60 seonds in TB60 group. All animals were resuscitated with lactated Ringer's solution (2 mL/100 g); fentanyl (0.1 mg/100 g) was used for analgesia.

Skin biopsies of the scrota and excisional biopsies of both testes from each rat were taken 1 hour after burn injury. The specimens were prepared for histological examinations. Animals were killed by high-dose ketamine HCl.

Histopathologic examination

Specimens were fixed in formalin and embedded in paraffin blocks. Several sections 4 μm thick were obtained from the paraffin-embedded blocks and processed with hematoxylin and eosin. All hematoxylin and eosin sections of skin biopsies were evaluated, and damage to the skin was classified as follows: epidermis normal or minimal damage (E1), moderate damage of epidermis (E2), severe damage of epidermis (E3), dermis normal or minimal damage

(D1), irregular collagen bands or mild homogenization of the dermis (D2), moderate-to-severe homogenization of the dermis (D3), destruction of dermal collagens (D4).

A standard 3-step immunoperoxidase avidin-biotin peroxidase complex technique was used to detect proliferating cell nuclear antigen (PCNA) (PC 10, Neomarkers, Fremont, CA, USA). To determine the average PCNA labeling index, approximately 1000 cells were counted for each case. The field to be counted was chosen under ×40 magnification from the well-labeled area. The PCNA labeling index (proliferation index) is expressed as a percentage ratio of total labeled cells to the total number of cells counted.

Death-associated DNA fragmentation in testis specimens was assessed in situ by terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) using a commercially available kit (ApopTag, Intergen, Purchase, NY, USA). After deparaffinization, tissue sections were washed for 5 minutes in phosphate-buffered saline (PBS), digested with proteinase K for 15 minutes at room temperature, and rinsed with distilled water for 2 minutes, 2 times. Slides were placed in 3.0% hydrogen peroxide for 5 minutes at room temperature to quench endogenous peroxidase activity and then rinsed twice with PBS for 5 minutes each time. An equilibration buffer (75 μL) was immediately applied to the specimens for 10 seconds at room temperature. TdT enzyme (33 μL) was mixed in the labeling buffer (77 μL). Excess liquid was removed, and the labeling reagent (20 μL/sample) was added. Slides were then placed in a humidified box and incubated for 1 hour at 37°C. A blocking solution was added to the slides for 10 minutes at room temperature. The blocking solution was then removed from the slides. The anti-digoxigenin conjugate was added to the slides, and the slides were kept in a humidified box, incubated at room temperature for 30 minutes, and then washed with PBS for 2 minutes, 4 times. DAB peroxidase substrate was applied to the slides. They were stained at room temperature for 3 to 6 minutes. For optimal staining, the reaction was monitored under a microscope.

After staining, the slides were rinsed with distilled water for 1 minute, 3 times. Sections were counterstained and permanently mounted. One hundred cells were counted, and the TUNEL index was expressed as the number of positive cells/total number of cells in the spermatogenic series.

RESULTS

On macroscopic examination, all burn wounds were limited to the groins and bilateral hemiscrotums of the animals in the T30, T60, and TB60 groups.

Immunohistochemical and histopathological findings

On histopathological examination of the scrotal skin, epidermal and dermal damage increased in parallel with the duration of contact and decreased with melaleuca hydrogel application: In the T30 group, epidermal destruction was grade 1 in 7 of the specimens (70%), and it was grade 2 in 3 (30%). In the T60 group, while 1 specimen (10%) in the whole group had an epidermal injury of grade 1, 7 (70%) had grade-2, and 2 (20%) had grade-3 injuries. In the TB60 group, epidermal destruction was grade 1 in 4 specimens (40%), and 6 specimens (60%) had grade-2 epidermal destruction. Dermal destruction in the T30 group was grade 2 in 7 specimens (70%), and it was grade 1 in 3 specimens (30%). In the T60 group, dermal destruction was grade 3 in 7 specimens (70%), and grade 2 in 3 specimens (3%). And in the TB60 group, 9 specimens had dermal destruction of grade 1, while only 1 had grade 2.

The means of the TUNEL index of apoptotic cells in spermatogenic series of the sham group, the T30 group, and the T60 group, were significantly different from each other; the highest TUNEL index was observed in the T60 group ($P < .05$). The mean apoptotic activity in the TB60 group was similar to that of the T30 group ($P > 0.5$). Proliferative indexes were similar in the sham, T30, T60, and TB60 groups ($P > 0.05$) (Table 1).

Coagulation necrosis and testicular degeneration in addition to apoptotic activity were increasingly observed in the T30 and T60 groups (Figure 1).

DISCUSSION

We demonstrated beneficial effects of immediately cooling thermally injured testes. Applying melaleuca hydrogel was preferred for cooling. A single application of melaleuca hydrogel is easy to do and is known to be as effective as repeated cold-water compresses (3). Melaleuca hydrogel can reduce elevated intradermal temperatures to below preburn levels within 6 minutes of application (3). Although temperature changes of the deep scrotum were unknown, we think that the cooling effects continued at least until the end of our observation period, and our findings suggest that application of melaleuca hydrogel minimizes the harmful effects of heat on the testes as well as on the scrotal surface.

An increase in epithelial cell growth has been noted with cooling of the skin after burn trauma (12). Our findings suggest that melaleuca hydrogel application caused a statistically significant decrease with regard to damage to the scrotal skin, but no increases were seen in the epithelial proliferative indexes in the testicular tissues of the entire study population. However, the observation period in the present study is not enough to understand if epithelial cell growth in testicular tissue is induced by cooling. Further studies with longer observation periods are essential to enlighten the issue.

On the other hand, in our previous studies of proliferative indexes and their relations to severe thermal injuries to the testes, we found that steam burns caused a failure of proliferation only when the testes

were in contact with steam for 90 seconds. More-severe thermal injury to the scrotal skin would cause more-severe spermatogenic cell injury and therefore, longer epithelization during the early postburn period (11). Our present data showed no negative or beneficial effects of cooling on the proliferation of spermatogenic cells. The effects of cooling in these more-severe cases warrants further investigation.

Apoptosis is known to be common in normal germinal epithelial cells and is believed to play an important role in controlling germ cell numbers and eliminating defective germ cells to produce functional spermatozoa (12). In addition, heat stress causes disruptions of mitochondrial membrane integrity and a release of cytochrome c. These events trigger the stimulation of caspase-9 and caspase-3 activity in the cytoplasm and induce abnormal apoptosis in the testes (13). There was a significant decrease in the apoptotic indexes of the testes to which melaleuca hydrogel had been applied (TB60 group) compared with the group that did not have melaleuca hydrogel applied (T60 group). The mean apoptotic index in TB60 group was lower than that of the T60 group and similar to that of the T30 group. We believe that cooling decreased the apoptotic indexes of spermatogenic cells in the TB60 group, which in turn decreased harmful aerobic metabolism in the tissues to which the applied melaleuca hydrogel had been applied (14-16). We think that in the presence of a cool environment, the integrity of mitochondrial membrane may be protected, and that release of cytochrome c from the mitochondria of spermatogenic cells might have been prevented to some degree (17). Therefore, aerobic metabolism in the cytoplasm is reduced by cooling. Further ultrastructural studies are needed to confirm the details of this proposed mechanism.

Furthermore, we suggest that cryotherapy (which also improves the tissue response to thermal injury by decreasing inflammatory and microvascular changes) might have had beneficial effects on the testicular response to heat. These effects could have been triggered by decreases in the release of histamine, prostaglandins, and thromboxanes (14, 16, 18). Thus, cooling and other methods or agents that enhance the tissue response to thermal injury must be the subject of future studies regarding the prevention of testicular thermal injury due to scrotal burns.

In conclusion, immediate cooling after burn trauma decreases testicular damage as measured by apoptosis and cellular degeneration in an experimental scrotal steam-burn model in rats. To prevent negative effects of heat to male reproductive functions in burn patients, the long-term effects of severe heat stress and its treatment must be investigated.

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