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Effects of Fluid Resuscitation on Testicular Damage in Severely Burned Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PIJ and IOF designed the study and wrote the protocol. Authors PIJ and JAO carried out the animal experiments and the histology work. Author PIJ wrote the first draft of the manuscript. Author ABO managed literature searches and edited the manuscripts. All authors read and approved the final manuscript.

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ABSTRACT

Background: Severe burns cause a wide range of complications that challenge care in the short term and often leaves long term sequelae in survivors. Research evidence suggests that burns can affect testosterone secretion, and impair spermatogenesis and fertility. This study was designed to investigate the effects of fluid resuscitation on spermatogenesis and histology of the testes after major burns.
Methodology: Third degree burns was induced on dorsal skin of 3 groups of male Wistar rats. Intra-venous resuscitation fluid was administered, ½ h and 3 h post-burn in the first 2 groups. A fourth group had sham burn only and served as negative control. After 8 weeks the animals were sacrificed evaluated for sperm parameters, testicular histology and assays of oxidative status.
Main Findings: Fluid administration did not cause significant difference in sperm parameters or in levels of markers of oxidative stress among the animals with burn injury. Mean sperm density in

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groups 1 and 2 which had fluid treatment were 57.00±11.99 and 56.33±9.49 respectively compared to 49.00±6.24 in the untreated group 3 (p=0.921 and 0.947). However total counts were significantly lower in all burned groups than in the sham burn group. Fluid treatment produced a time-dependent relief from the histological disruptions associated with the burns. Tubules with germ cell loss were fewer in the fluid treated groups than in the untreated one.

Conclusion: Fluid treatment in burn patients may not protect them from suppression of testicular function. Fertility damage in severe burns may involve mechanisms that do not depend on intravascular volumes and pressures.

Keywords: Spermatogenesis; burn injury; resuscitation fluid; sperm parameters.

1. INTRODUCTION

Despite significant reduction in burn mortality over the past few decades, burn injuries remain a major health hazard worldwide. Burns cause pathophysiological alterations in almost all body tissues. The body responds to major burns with a prolonged hypermetabolic state characterized by raised resting energy expenditure, raised oxygen consumption and carbon dioxide production. These changes are associated with increased nitrogen mobilization and excretion, leading to tissue wasting and a fall in lean body mass [1,2].

Severe burns also produce a shift in emphasis in the endocrine system from making sex steroid hormones to producing stress hormones such as cortisol which is now believed to drive the inflammatory response to burns [3,4]. Several organs are affected by the prolonged hypermetabolic response. Among mammals, considerable germ cell loss has been demonstrated in rats subjected to burns, especially when wound healing was complicated by prolonged debility [5,6]. Chronic debility from any disease state can lead to a fall in serum testosterone levels and thereby impair spermatogenesis [7,8].

We have previously shown that in human survivors of severe burns, mean sperm count was suppressed [9]. We also showed that there was a significant correlation between sperm count and the time lag from burn injury to onset of medical treatment [10]. In the low and medium income countries where the vast majority of the world's severe burn injuries occur, patients still reach hospital after several hours delay. In this study we investigated the hypothesis that hypo-perfusion during this time lag is a major contributor to testicular damage observed after burns. We did this by varying the time lag between burns and the onset of fluid administration in a rat model. We also examined the probable effects of reperfusion injury on the pathophysiology of post-burn infertility in male rats.

2. MATERIALS AND METHODS

2.1 Treatment Groups

Twenty (20) Wistar rats weighing between 200 – 250 g were obtained from a stock bred at the Animal House of our Medical College. They were fed standard rat chow and given clean water ad-libitum. They were acclimatized in our laboratory for one week.

Intra-venous fluids, ketamine and xylaxine were bought from a local pharmacy in Lagos. All other chemicals were of pharmaceutical grade. The animals were randomly assigned into one of four experimental groups and treated as follows:

GP 1 3rd degree burns + 120 ml/kg of treatment fluid ip, 30 min post-burn.
GP 2 3rd degree burns + 120 ml/kg of treatment fluid ip, 3 hours post-burn.
GP 3 3rd degree burns with no treatment fluid post-burn.
GP 4 Only sham burn was applied.

2.2 Animal Treatment

In groups 1 to 3 uniform 3rd degree burn injury was induced on 35% of skin on the dorsum of the animals by a method previously described [5]. Briefly, skin on the dorsum of the rat was shaved under ketamine anaesthetics, and the skin was exposed to scalding water at 99ºC for 10 seconds. In group 4, the animal was shaved and the skin exposed to water at room temperature. The calculated resuscitation fluid was divided into two equal parts. One part was given from 4.3% Dextrose in saline, and the other from lactated Ringers solution. The fluid was given by slow intra-peritoneal injection in a single bolus. The animals were kept in single cages in 12

hours light/darkness rhythm. The study design was approved by our Institution's Committee on the Care and Use of Experimental Animals and complied with the Helsinki declaration on handling of experimental animals.

2.3 Termination of Experiment

The animals were sacrificed at the end of 8 weeks. The testes and accessory sex organs were dissected out, freed of fat and weighed. The caudal part of the epididymis was used for estimating sperm parameters. One testis was fixed in Bouin's fluid for histological studies. The other testis was macerated in distilled water and used to measure biochemical parameters.

2.4 Determination of Experimental Sperm Parameters

Epidydimal sperm concentration, motility and morphology were determined by methods already described elsewhere. Briefly, the cauda epidydimis was minced in fresh 2 ml of physiologic saline solution buffered with sodium bicarbonate and allowed to stand for a few minutes to liberate spermatozoa. Motility estimation was carried out at room temperature between 24ºC and 28ºC. 1ml from the sperm suspension was placed on a warmed slide and the microscopic field was scanned systematically. Spermatozoa encountered were assessed as motile or non-motile. An estimate of the percentage of motile sperm was made [11]. Sperm density was estimated by charging a haemocytometer from a preparation made by diluting the sperm suspension 1:1 with distilled water to which a drop of formalin had been added to immobilise sperm. Sperm morphology was determined by counting all abnormal cells out of 100 cells examined across several high power fields [11].

2.5 Biochemical Assays

Malondialdehyde (MDA) levels were determined using a method described previously, and the resulting colour complex was measured at 532 nm because it absorbs light maximally at this wavelength. The result was expressed as nmol/mg protein in the sample [12].

Catalase (CAT) activity was determined from the rate of disappearance of H2O2 at 37ºC, measured colorimetrically at 620nm according to the method described by Rukmini and others and is expressed as u/mg protein in the sample [12].

Reduced glutathione (GSH) was estimated by the method of Ellman. Briefly, 0.5 ml of Ellman,s reagent was added to 1ml of the supernatant. Distilled water and 3ml of 0.2 M phosphate was then added to the mixture and the absorbance was read at 412 nm. The result was expressed as nmol/mg protein [13].

2.6 Histology

Tissue sections from testes and epidydimis were prepared for histological examination by the method of Sheehan and Wrapchak [14]. Briefly the specimens were cleared of fixative (Bouin,s fluid), blocked out in paraffin, processed and cut into 5µ sections. The sections were stained with hematoxyline and eosin and examined in a Ceti microscope fitted with an XLI camera and software at total magnifications ranging from 100 to 400.

3. RESULTS

3.1 General Observations

Animals in all groups tolerated the experiment well. They gained weight during the course of experiment. However topical silver cream (sulfadiazine) was applied occasionally to healing wounds in some animals to control itching. There was no significant difference in weight change between experimental groups. There was also no significant difference in weight change between experimental groups and control group. Weight and volume of testes and accessory sex organs did not show any significant differences between experimental groups or between them and control group (Table 1).

3.2 Sperm Parameters

Fluid administration did not cause significant difference in sperm parameters between experimental groups. Mean sperm density in groups 1 and 2 which had fluid treatment were 57.00±11.99 and 56.33±9.49 compared to 49.00±6.24 in the untreated group 3 ($p= 0.921$ and 0.947) respectively (Table 2). There was a significant difference in total counts and sperm motility between all experimental groups and control group ($p<0.05$). Significant difference only occurred between groups in the form of reduced motility in group 2. No significant difference was

observed in the percentage of cells with abnormal sperm morphology among groups.

3.3 Biochemical Parameters

Anti-oxidant enzyme levels – CAT and GSH were up-regulated but not significantly in the burned groups when compared to control. They were highest in group2 which had delayed fluid administration. Lipid peroxidation was higher in burned groups as well though in group I which had early fluid injection, it was lower than in control group. However when MDA levels are compared between groups, the levels in groups 2 and 3 were double and almost treble that of group I (Table 3) suggesting that early fluid administration reduced lipid peroxidation.

3.4 Histology

Burn injury caused disruption in the histology of testes in all experimental groups, irrespective of treatment. The predominant change was germ cell loss affecting mostly the more mature members of the germ series. Tubules with germ cell atrophy were fewer in fluid treated groups than in the untreated group however. Interstitium and tubule outline were generally intact. One observation in this study was that tubular atrophy was more pronounced in the periphery of the testes suggesting that blood-borne agents might be active in producing this effect (Fig. 3).

4. DISCUSSION

In this study sperm density was significantly lower in all burned groups than in control animals. Sperm motility was significantly lower in group 2 which had delayed fluid treatment than in all other groups. This shows that fluid treatment, when delayed provides no significant protection against injury to spermatogenesis. Reduced sperm motility in this group suggests that the parameter was probably negatively affected by reperfusion. Low oxygen tension and perfusion pressures are typical of the vascular bed in mammalian testes [15]. This partly explains why conditions associated with vascular congestion and raised pressures such as occurs in testicular varicoceles impair spermatogenesis and fertility in humans [16,17].

Animal studies in which the testicular artery was temporarily occluded by torsion and re-opened by de-torsion after scheduled time periods show that testicular function and histology was damaged when reperfusion was delayed beyond a critical threshold [18,19]. Several mammalian organs are known to be susceptible to reperfusion injury [20,21].

Table 1. Weight of sex organs and testis volume in all groups

Organ	Gp1	Gp2	Gp3	Gp4
Testis weight (g)	1.23±0.32	1.33±0.22	1.36±0.22	1.28±0.28
Testis vol.(ml)	1.18±0.03	1.10±0.02	1.30±0.04	1.15±0.03
Epidydimis wt (g)	0.28±0.08	0.29±0.07	0.28±0.07	0.31±0.06
Prostate wt (g)	0.36±0.08	0.53±0.11	0.35±0.09	0.52±0.12
SV wt (g)	1.4±0.24	0.53±0.22	1.06±0.25	1.53±0.31

Results represent the mean ± SEM. In paired organs, observations are mean of left and right organs. SV is seminal vesicle

Table 2. Epididymal sperm parameters in all groups

Parameters	Gp1	Gp2	Gp3	Gp4
Total count (m/ml)	57.00±11.9[a]	56.33±9.49[a]	49.00±6.24[a]	98.18±7.24
Motility (%)	57.5±4.77[a]	35.00±3.63[ab]	56.66±4.66[a]	82.33±1.45
Abnormal rate (%)	22.00±4.5	13.50±3.10	21.60±5.2	22.20±3.50

Results represent the mean ± SEM
a:Indicates significant difference when compared with control.
ab: Indicates significant difference between groups

Table 3. Anti-oxidant parameters in all groups

Parameters	Gp1	Gp2	Gp3	Gp4
MDA	0.03±0.01	0.06±0.02	0.08±0.03	0.05±0.01
GSH	0.15±0.03	0.10±0.01	0.08±0.00	0.09±0.01
CAT	15.75±1.34	17.68±0.60	14.65±0.09	17.66±0.61

Results represent the mean ± SEM

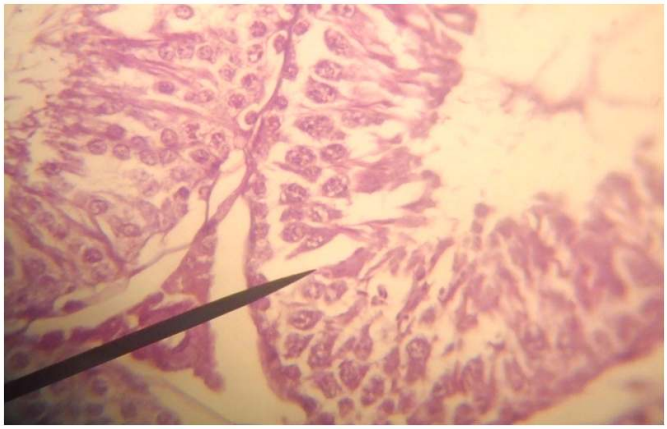

Fig. 1. Typical micrograph from group 1 animal
Note the appearance of ad-luminal cell lysis in both tubules and sloughing at the arrow in this profile. H&E, (original magnification: ×400)

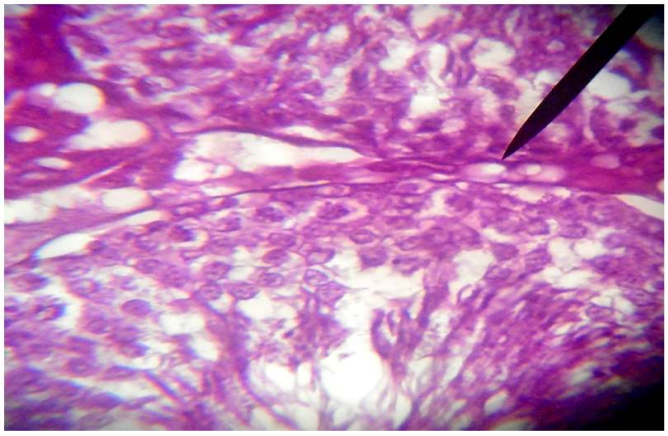

Fig. 2. Typical micrograph from group 2 animal
The tubule bellow the arrow shows sloughing, indicating germ cell atrophy. Interstitium is normal. H&E, (original magnification: ×400)

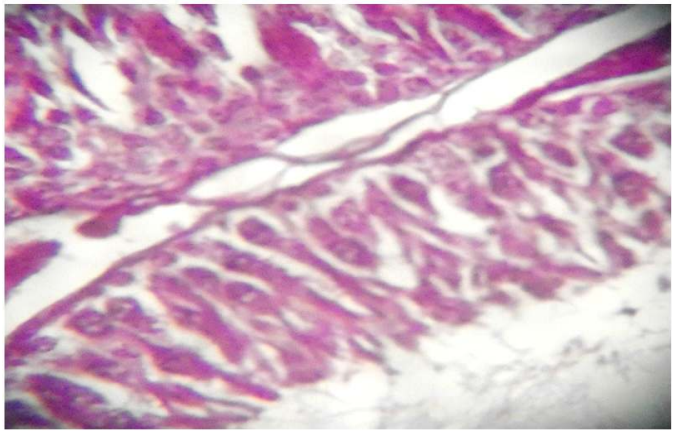

Fig. 3. Micrograph from group 3 animal
Many tubules showed considerable ad-luminal cell atrophy with only tail debris visible in the lumen. H&E, (Original magnification: ×400)

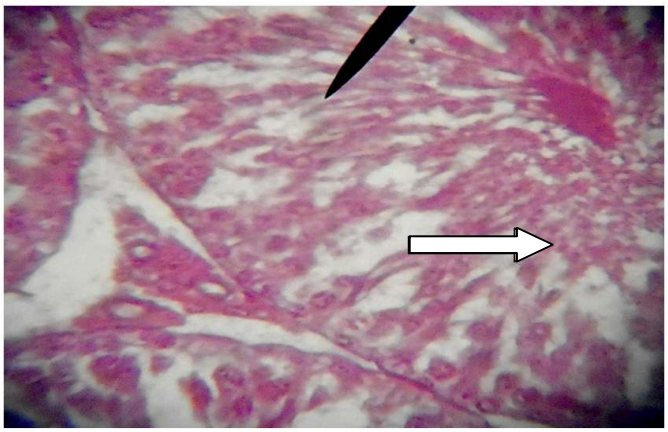

Fig. 4. Typical micrograph from group 4 animal (control)
Note normal cell arrangement and the lumen (white arrow) full of free mature sperm cells. Interstitium is intact. H&E, (original magnification: ×400)

In this study lipid peroxidation measured by MDA level was lowered by 50% in the early treatment group compared to groups 2 and 3 which had late and no treatment respectively. However anti-oxidants CAT and GSH showed no significant differences between groups. An important implication of these findings includes the fact that major burns may impair testicular function even when maintaining adequate vascular perfusion and anti-oxidant defenses.

These findings cannot however imply that anti-oxidant treatment have no benefit in burn patients as we have previously shown that ascorbic acid administration protects the testes from many of the deleterious effects of burns. The apparent contradiction may be explained by the fact that the assessments of tissue enzymes from macerated testes include molecules in the extracellular compartment and may not fully reflect the in-vivo situation where reactive oxygen species are being generated from intra-cellular processes.

In this experiment burn injury caused a pattern of histological alteration typical of what was reported in previous studies [5,6]. The typical finding in all burned groups was germ cell loss with a considerable number of tubules showing no free mature spermatozoa in their lumen. Evidence of histological damage was most severe in groups 2 and 3 where fluid treatment was either delayed or withheld. In these groups, almost 20% of tubules showed evidence of significant ad-luminal germ cell loss when compared to control group. Again early fluid treatment conferred some degree of protection from histological damage.

These finding are consistent with those from previous studies except that in cases where wound healing was delayed for long period much more severe damage occurred in the seminiferous epithelium [5,6].

This study assessed the possibility of early fluid administration protecting the testes from functional and histological damage induced by thermal injury. Our results show that in severe burns, fluid administration even when given within half an hour of injury was unable to provide statistically significant protection from damage to spermatogenesis and the histological integrity of the testes in rats. These findings highlight the evidence from much of the recent and especially basic burn research that severe burns induce extremely complex changes in the pathophysiology of the victim. These changes are usually induced by inflammatory, endocrine, immune and cytokine influences. It is unlikely that any single therapy can possibly address the multiple points at which pathological changes are taking place in these patients. This complexity of pathology partly explains why paradoxical results sometimes follow clinical trials even when they were based on evidence from basic research in burns. These paradoxes in turn contribute to the considerable gap between basic research in burns and their clinical applications. Research however needs to continue until the unique combination of therapies is found that addresses the many points at which tissue injury is taking place in the burn victim.

5. CONCLUSSION

Maintaining stable heamovascular conditions through fluid therapy is a key early goal in managing burn patients. While this practice reduces mortality from shock and the occurrence of some of the later complications of burns, our findings in this study show that fluid therapy, even when given promptly may not be sufficient to prevent testicular damage from this injury. More research is needed to develop a package of measures that addresses the myriad of pathologic insults that damage the testis and suppress spermatogenesis in these patients.

CONSENT AND ETHICAL APPROVAL

No human subjects were used in this study and consent does not apply. All authors hereby declare that the study protocol for this study was approved by our Institution's Ethics Committee and complied with the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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