Research Article

Exogenous Ascorbate Administration Elevates Testicular Oxidative Damage and Histological Injuries in Rats after Busulfan Treatment

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Several anticancer drugs are coadministered with ascorbate (ASCB) to complement their cytotoxic effects. However, it is not known if the treatment regimen prevents collateral oxidative damage to non-target sites. The current study evaluated the effect of ASCB cotreatment on the testes of young adult rats treated with an anticancer drug, busulfan (BUS). About 40 Wistar rats were arbitrarily assigned into four groups ($N = 10$), namely, control (<0.2% dimethyl sulfoxide vehicle), BUS (4 mg/kg b.w.; intraperitoneally for 4 days), BUS + ASCB (4 mg BUS/kg b.w. for 4 days + 100 mg ASCB/kg b.w.; intraperitoneally for 14 days and 7 days prior to start of BUS injection), and ASCB (100 mg/kg b.w.; intraperitoneally for 21 days). At the end of study, ASCB + BUS cotreatment reduced spermatogenesis score index, superoxide dismutase activity, total ASCB and decreased hydrogen peroxide level, and elevated catalase activity and nitrite concentration much more than treatment with BUS alone ($P < 0.05$). Other observations included elevated malondialdehyde level, DNA damage, and diminished glutathione concentration in the testes of BUS + ASCB animals. Interestingly, ASCB administration raised testicular ASCB concentration beyond the control values ($P < 0.05$) and the antioxidant status of the testes. Histological aberrations included many single layers of germ cells, shrinked tubules, and vacuolated structures in the epithelium of BUS + ASCB, similar to those of BUS-treated animals. In conclusion, BUS treatment deregulated the redox status of the testes and caused a dramatic consumption of ASCB which were enhanced by exogenous ASCB resulting to testicular damage.

1. Introduction

Busulfan (1,4-butanediol dimethanesulfonate) belongs to the alkyl sulfonate class of anticancer drugs that are commonly used in the treatment of lymphoma and leukemia and as a preparatory agent before grafting of blood stem cells [1]. It is a common knowledge that the treatment of leukemia with busulfan (BUS) in young patients could cause oligospermia, including azoospermia and testicular damage with germ cell loss that lead to infertility in certain cases, especially over a long period of application time [2]. The use of anticancer drugs, including BUS in cancer patients, is identified as risk factors for male-factor infertility [3–5].

In animal models, BUS administration preferentially damages stem cells spermatogonia, disrupts spermatogenesis, impairs sperm cell morphology, and destroys the seminiferous epithelium [1] and when the treatment is extended for a long time, it eliminates sperm cells from the testes and decreases sperm count and causes infertility [1, 6]. Pregnant animals were found to produce offspring with germ cell-free gonads after treatment with BUS alone ($P < 0.05$). Other observations included elevated malondialdehyde level, DNA damage, and diminished glutathione concentration in the testes of BUS + ASCB animals. Interestingly, ASCB administration raised testicular ASCB concentration beyond the control values ($P < 0.05$) and the antioxidant status of the testes. Histological aberrations included many single layers of germ cells, shrinked tubules, and vacuolated structures in the epithelium of BUS + ASCB, similar to those of BUS-treated animals. In conclusion, BUS treatment deregulated the redox status of the testes and caused a dramatic consumption of ASCB which were enhanced by exogenous ASCB resulting to testicular damage.
lipid peroxidation, and intracellular oxidative stress [15, 16]. Moreover, BUS-mediated ROS production, oxidative damage, and decrease in testicular antioxidant capacity lead to severe damage to the reproductive tissues [5, 13, 16] and loss of spermatogenesis and fertility in animal models [14].

L-Ascorbic acid (ascorbate) is a vital water-soluble micro-nutrient crucial for variegated biological functions. It behaves as chain-breaking antioxidant both in the plasma and within cells and tissues, reduces free radical generation, inhibits lipoperoxidation and oxidative stimuli induced by chemical agents and environmental factors, increases testosterone level in serum, protects spermatogenesis, and promotes semen quality and fecundity in human and animals [17–21]. L-Ascorbic acid also yields favorable effects on the body defense system and inflammatory reactions, which is why it is used in chemotherapy to halt premalignant and cancer cells by the host [22]. Interestingly, treatment with anticancer agents leads to low vitamin C plasma level either as a consequence of reduced dietary intake or increased requirement for ascorbate (ASCB) in diseased cells, for example, malignant cells [19, 23, 24].

Although, numerous animal species can produce ascorbic acid, humans cannot and are consequently dependent on intake through diet or as a supplement of vitamin C-containing medicines. The efficacy of ASCB in the biological system revolves around its redox capacity in diverse enzymatic reactions. In physiological concentrations, it acts as an antioxidant and at higher doses, it has proxidant effects [22]. There are numerous assumptions concerning the way ASCB exerts pharmacological effects in tissues and cells. At pharmacological concentrations, vitamin C activates the formation of H$_2$O$_2$. The H$_2$O$_2$ can form toxic ROS that directly kills the cancer cells [25]. Some investigators have also shown that the L-Ascorbic acid can enhance the effect of chemotherapy in preclinical model systems [19, 26].

The fact that majority of cancer patients, treated with anticancer drugs are predisposed to fertility health issues, have raised the necessity for study on the treatment of chemotherapy-related gonadal toxicity during cancer [6, 9–12, 27]. L-Ascorbic acid has been well explored as a constituent part of a treatment regimen, either owing to its synergistic effect with chemotherapy or, surprisingly, cancer cell death suppression agent [20]. At physiological levels, L-Ascorbic acid behaves as an antioxidant whereas its pharmacological efficacy is connected to its pro-oxidant effects, and consequently, promotes the killing of cancer cells [28]. Nevertheless, the action of ASCB on the testis during chemotherapy has not been recognized. This study was undertaken to determine if gavage administration of ascorbic acid could protect the testes of a normal healthy rats against BUS toxicity. We report findings of the work by Ayyildiz et al. [29] which measured the effect of L-Ascorbic acid at dissimilar doses (25, 50, 100, 200, 400, or 800 mg/kg) and found that 100 mg/kg body weight was the most effectual dose. This has also been demonstrated by other investigators as the most plausible protective agent in animal models of testicular injuries [30].

2. Material and Methods

2.1. Chemicals. BUS and ascorbic acid and all other reagents used in this study were obtained from Sigma–Aldrich St. Louis, Missouri (USA) unless otherwise stated. BUS was prepared in dimethyl sulfoxide and ASCB in normal saline and use throughout the experiment. The final concentration of dimethyl sulfoxide vehicle was not more than 0.2%.

2.2. Experimental Design. About 40 young adult male Wistar rats weighing 90 + 17 g were purchased from a rat breeder in Choba community of Rivers State. They were allowed 2 weeks to acclimatize to their surroundings at the animal house of the Department of Biochemistry and kept under the same ambient conditions concerning food, fresh drinking water and light (12L:12D cycle) in conformity with the standard basis of Laboratory Animal Care. The rats were randomly sorted into four groups (N = 10): control (received dimethyl sulfoxide vehicle; <0.2%), BUS group was injected 4 mg BUS/kg body weight (b.w.) intraperitoneally, ASCB group was injected 100 mg ASCB/kg b.w. intraperitoneally, and BUS + ASCB group received both BUS and ASCB in the same manner and doses as in the single treatment groups. ASCB was coadministered daily for 14 days and BUS for 4 days daily. Treatment with ASCB started 1 week earlier than BUS injection and continue for 2 weeks when the experiment was terminated. The 14-day duration is ideal for observable effects of BUS on the morphology of the testis of animal models [9–12]. The BUS dose (4 mg/kg/day) was adopted on the premise of our earlier study that demonstrated testicular injury with no significant mortality of the animals [10]. The tested dose of ASCB was equally designated based on the findings of the work by Ayyildiz et al. [29] which measured the effect of L-Ascorbic acid at dissimilar doses (25, 50, 100, 200, 400, or 800 mg/kg) and found that 100 mg/kg body weight was the most effectual dose. This has also been demonstrated by other investigators as the most plausible protective agent in animal models of testicular injuries [30].

2.3. Preparation of Tissue Homogenates. After sacrifice of animals, the right tissues were detached and put in cold 1.15% potassium chloride for the removal of blood stains. They were cut into bits and homogenized in ice-cold sodium phosphate buffer, pH 7.5 (0.1 M) using mortar and pestle that were placed in a tray of ice. Centrifugation was done at 10,000 revolutions per minute at 4°C for 30 min to generate the supernatant fraction that was immediately used for the biochemical assays. Protein concentrations of the samples were determined according to the procedure described by Lowry et al. [31].

2.3.1. Determination of Lipid Peroxidation Level. Lipid peroxidation level was determined as previously described by Ohkawa et al. [32]. The absorbance of the solution was read at 532 nm and was used to calculate malondialdehyde (MDA) concentration using the extinction coefficient of thiobarbituric acid reactive substances and expressed as µmol MDA ml$^{-1}$ mg protein$^{-1}$.

2.3.2. Determination of Catalase Activity. Catalase activity was measured as described by Chance and Maehly [33]. The absorbance change per minute at 240 nm was defined as one unit of catalase activity and expressed as units mg protein$^{-1}$ ml$^{-1}$.
2.3.3. Determination of Reduced Glutathione Level. Protein-free samples were mixed with freshly prepared Ellman's reagent (10 mM) as previously described by Moron et al. [34]. The mixture was incubated at room temperature for 20 min in the dark to allow for the formation of a light yellow solution. The absorbance of the solution was measured at 412 nm and concentration of glutathione (GSH) was calculated as μg mg protein⁻¹ ml⁻¹.

2.3.4. Determination of Superoxide Dismutase Activity. Superoxide dismutase enzyme activity was determined as previously reported by Kakkar et al. [35]. The absorbance of the dark-blue solution formed after addition of reduced nicotinamide adenine dinucleotide (NADH) was recorded at 560 nm. The activity of superoxide dismutase was expressed in nmoles NADH mg⁻¹ protein ml⁻¹.

2.3.5. Determination of Nitrite Concentration. Nitrite concentration was determined by the Griess assay [36]. The sample was mixed with Griess reagent and incubated at room temperature in the dark for 15–20 min to allow for the formation of a light-pink solution. The absorbance of the solution was recorded at 540 nm and the concentration of nitrite oxide was extrapolated from a sodium nitrite calibration curve (0–80 μM) and expressed as μmol per mg of protein/ml.

2.3.6. Determination of H₂O₂ Generation. Hydrogen peroxide generation in the sample was assayed based on the ferrous oxidation with xylenol orange (FOX-1) as described by Wolff [37]. The assay mixture was allowed to stand at room temperature for 45 min for the formation of pink solution. H₂O₂ concentration was calculated by recording the absorbance at 560 nm using a standard curve (10–100 μmol) for H₂O₂. The concentration of H₂O₂ was expressed as μmol of H₂O₂ per mg of protein/ml.

2.3.7. Determination of Total ASCB Concentration. Total ASCB level (oxidized plus reduced ASCB) was assessed by the 2,4-dinitrophenyhydrazine reaction according to the protocol of Omaye et al. [38]. The concentration of ascorbic acid was measured from the absorbance of the reaction mixture at 520 nm using ascorbic acid standard curve (0–80 μg/ml). The concentration was expressed as μmol of H₂O₂ per mg of protein/ml.

2.3.8. Quantification of Percentage Fragmented DNA by Diphenylamine Reaction. DNA fragmentation was quantified as described by Sandau et al. [39]. After overnight incubation, the supernatant and pellet fractions were assayed for DNA content by diphenylamine reaction at 600 nm and expressed in percentages.

2.4. Histopathological Examination of Tissues under Light Microscopy. The harvested testes were fixed in Bouin’s solution for 24 hr. Bouin’s fixed samples were routinely dehydrated in ascending ethanol series, encased in paraffin wax, and sectioned at 5 μm thick on glass slides. The sectioned tissue blocks were stained with hematoxylin and eosin and viewed using a light microscope at 400x magnification.

2.5. Spermatogenesis Score Index and Seminiferous Tubular Diameter. To evaluate Johnsen’s score, 25 round or nearly circular seminiferous tubules were randomly selected from transverse sections of testes, and the spermatogenesis score index (scale of 1–10 based on the degree of spermatogenesis) was measured for each tubule [40] and computed as the average Johnsen’s score. The seminiferous tubular diameter was analyzed in 50 randomly selected circular or nearly round seminiferous tubules for each animal captured at 100x magnification using the straight-line tool of Image J software (National Institute of Health, Bethesda, MA, USA).

3. Results

3.1. Antioxidant Marker Enzymes (Superoxide Dismutase and Catalase) and Hydrogen Peroxide Concentration in the Testes of Rats after BUS and ASCB Cotreatment. Superoxide dismutase activity was found to decrease after BUS treatment. The decreased activity was lower by 42% compared to BUS values after cotreatment with ascorbic acid (P < 0.05) (Figure 1(a)). Superoxide dismutase activity after ascorbic acid coadministration was lower than the control values and ASCB (P < 0.05). Treatment with ASCB increased superoxide dismutase activity relative to the control values. H₂O₂ generation was decreased after BUS treatment (P < 0.05), and the decreased H₂O₂ level was sustained after ascorbic acid cotreatment (BUS + ASCB) compared to the BUS values, even when ascorbic acid treatment alone elevated H₂O₂ concentration compared to the control values (P < 0.05) (Figure 1(b)). Catalase activity was increased by BUS treatment. The elevated catalase activity was sustained after cotreatment with ascorbic acid (BUS + ASCB) (Figure 1(b)) compared to the BUS values (P < 0.05). Furthermore, catalase activity after ascorbic acid administration remained unchanged versus control values (P > 0.05) (Figure 1(c)).

3.2. Lipid Peroxidation and GSH Concentration in the Testes of Rats after BUS and ASCB Cotreatment. Lipid peroxidation was escalated after BUS treatment compared to the control values (P < 0.05) (Figure 2(a)). After ascorbic acid coadministration, lipid peroxidation was also high in the testes of the BUS + ASCB animals. In fact, MDA concentration (μmol MDA mg protein⁻¹ ml⁻¹) in the testes of these animals was 14% higher than those of the BUS group; although the change did not get to statistical significant level (P > 0.05).

Treatment with ascorbic acid alone did not alter MDA concentrations in the testes of the rats when related to the
FIGURE 1: Effects of ascorbic acid pretreatment on (a) superoxide dismutase activity, (b) hydrogen peroxide concentration, and (c) catalase enzyme activity in the testes of busulfan-treated rats. Data (N = 10) are mean ± SD and were analyzed by ANOVA and Tukey’s post hoc test. Superoxide dismutase activity and hydrogen peroxide concentration = *versus control; **versus BUS; catalase enzyme activity = *versus control; **versus ASCB; P < 0.05. N, sample size per each group; ASCB, ascorbate; BUS, busulfan.

FIGURE 2: Effects of ascorbic acid pretreatment on (a) lipid peroxidation levels and (b) reduced GSH concentration in the testes of busulfan-treated rats. Data (N = 10) are mean ± SD and were analyzed by ANOVA and Tukey’s post hoc test. *Versus control; P < 0.05. N, sample size per each group; ASCB, ascorbate; BUS, busulfan; GSH, glutathione.
control values \((P>0.05)\) (Figure 2(a)). GSH concentration \((\mu \text{g GSH mg protein}^{-1} \text{ml}^{-1})\) in the testes of BUS-treated rats was reduced by 44% compared to the control \((P<0.05)\). Coadministration of ascorbic acid \((\text{BUS} + \text{ASCB})\) did not ameliorate this decrease compared to the BUS group. In fact, GSH level in this group \((\text{BUS} + \text{ASCB})\) of animals was decreased compared to the BUS group by 13% \((P>0.05)\), suggesting that the decrease in GSH level after BUS administration was sustained after coadministration of ASCB. Interestingly, ascorbic acid administration alone to the ASCB group of animals could increase GSH concentrations by 65% relative to the control (Figure 2(b)).

3.3. Nitrite Concentration in the Testes of Rats after BUS and ASCB Cotreatment. Nitric oxide was quantified as nitrite in the testes of treated animals because of its extremely short physiological half-life [42]. After BUS treatment, nitric oxide was increased, and the increase was not attenuated after coadministration of ascorbic acid (Figure 3). In fact, nitric oxide was elevated by 10% after ascorbic acid coadministration compared to the values of the BUS group \((P<0.05)\). Interestingly, ascorbic acid treatment alone could increase nitric oxide level by 150% compared to the values of the control \((P<0.05)\).

3.4. Total ASCB Concentration in the Testes of Rats after BUS and ASCB Cotreatment. Total ASCB concentration \((\mu \text{g ASCB mg protein}^{-1} \text{ml}^{-1})\) was decreased compared to the values of the control group \((P<0.05)\) after BUS treatment. Coadministration of ascorbic acid decreased ascorbic acid concentration in the testis by 46% compared to the BUS group \((P<0.05)\). Furthermore, ASCB concentration in the testes of rats after treatment with ascorbic acid alone was increased by 53% relative to the control values \((P>0.05)\) (Figure 4).

3.5. DNA Fragmentation in the Testes of Rats after BUS and ASCB Cotreatment. BUS treatment increased the percentage of fragmented DNA in the testes of treated rats \((P<0.05)\) when compared to the control values. The rise in DNA fragmentation was also found in the testes of the animals after cotreatment with ascorbic acid \((\text{BUS} + \text{ASCB})\) compared to control values \((P<0.05)\). The changes in the percentage fragmented DNA when the BUS values were compared to the BUS + ASCB group of animals did not reach statistically significant levels \((P>0.05)\). Furthermore, after ascorbic acid administration alone, the percentage fragmented DNA remained unchanged in the ASCB group when compared to the control values \((P>0.05)\) (Figure 5).

3.6. Light Microscopy of the Testes of Rats after BUS and ASCB Cotreatment. The testes of control rats had well-defined lumen, many layers of well-arranged germ cells (spermatogonia, spermatocytes, and spermatids) and Sertoli cells located toward the basement membrane, intact epithelium,
packed with germ cells at divergent developmental stages, and tufts of spermatozoa seen toward the luminal area and interstitial cells in the intertubular areas. The histological facets of the testes section of BUS-treated animals and those cotreated with ASCB have single layers of germ cells which consist mostly of spermatogonia. Sertoli cells were found toward the basement membrane. Several vacuolated structures were also seen in the epithelium, and the tubules were farther apart such that the intertubular areas were wider and filled with interstitial cells, including Leydig cells. Some tubules with germ cells were degenerated, atrophied, and disorganized in the ASCB-cotreated rats. The histological features of the testes of the ASCB-treated rats were not different from the control animals and had no obvious morphological defects and histologic aberrations (Figure 6).

**3.7. Spermatogenesis Score Index and Seminiferous Tubular Diameter of the Testes of Rats after BUS and ASCB Cotreatment.**

As expected, BUS treatment decreased spermatogenesis as reflected by the reduced Johnsen’s scores when compared to the control values ($P<0.05$). Cotreatment of ASCB decreased the spermatogenesis score index by 28% when compared to the values found in the BUS-treated animals ($P<0.05$). In addition, the decrease in the degree of spermatogenesis in the BUS group was 67% compared to the 76% observed in the BUS + ASCB-treated animals when they were compared to the control values. Treatment with ASCB alone did not change the Johnsen’s score when compared to the control values (Figure 7(a)). The seminiferous tubular diameter was decreased after BUS treatment and when cotreated with ASCB ($P<0.05$) compared to the control values. Treatment with ASCB did not alter tubular diameter relative to the control values (Figure 7(b)).

### 4. Discussion

We showed in the present study that BUS administration inhibited superoxide activity and H$_2$O$_2$ concentration and increased catalase activity in the testes of the young healthy rats. In tissues, superoxide dismutase reduces superoxide radicals to the less toxic hydrogen peroxide which is thereafter converted to water and oxygen by catalase [43]. This detoxification pathway ensures that two possibly injurious species, superoxide radicals and hydrogen peroxide, and other toxic oxygen metabolites generated during BUS metabolism in the testis of the animals are inactivated and converted to water. The resulting oxidative stress is therefore associated with the altered redox equilibrium. To compensate for the low activity of superoxide activity induced by BUS administration, catalase activity was found to increase resulting to the decrease in H$_2$O$_2$ concentration. Furthermore, the elevated oxidative stress after BUS application was manifested as increased MDA concentration and accompanied with reduced GSH concentration in the testicular homogenates. These observations are similar to what happens in tissues undergoing oxidative stress after exposure to oxidant drugs [8, 44]. GSH is highly expressed in testicular tissues of which they serve as major antioxidants in the testes and buffers the cells against exogenously induced oxidative stress. It is known that BUS could conjugate with GSH [44], thus increasing oxidative stress. Therefore, the decrease in GSH and increase in lipid peroxidation in the present study.
is synonymous to oxidative stress in the testis. There are several literatures that have reported similar effect of BUS in animal models as reported in the present study, and it is now accepted that BUS-induced oxidative stress is linked with testicular insufficiency due to altered antioxidant defense status that leads to lipid peroxidation [6, 8–12].

The mechanism of the detrimental effect of BUS can also be attributed to its direct toxic effects on the testes [12, 45]. The present study further showed that BUS could reduce spermatogenesis as reflected by the absence of the round spermatids. In addition, the tubules had only Sertoli cells and a few spermatogonia cells lining the basement membrane. These findings have been supported in several previous studies [9–12, 14, 46–48]. In addition, tubular diameter and Johnsen’s scores were also decreased in the testes of BUS-treated rats in the present study. Other studies showed that the few germ cells in the tubules were due to the fact that BUS promoted the killing of germ cells by oxidative damage resulting to the halting of subsequent stages of spermatogenesis and maturation arrest [6, 10, 14, 45, 49]. Our results are in agreement with most of these studies and confirm the role of BUS-induced oxidative stress in the observed testicular injury and impaired spermatogenesis. Therefore, the inhibition of spermatogenesis and diminished tubular diameter in the BUS group in this study are the result of the altered antioxidant status in the testes of animals which can lead to poor sperm quality [45].

We further showed in the present study that BUS treatment increased nitric oxide level and DNA fragmentation and decreased ASCB concentration in the testes of rats. DNA fragmentation in the sperms of BUS-treated mice has been reported by Nasimi et al. [6]. In our earlier study, we demonstrated an increase in nitrite concentration in the testes of BUS-treated rats [10, 11], thereby supporting our present findings. The decrease in testicular ascorbic acid concentration after BUS treatment further confirms that oxidative stress was induced in the testes [50]. Oxidative stress could result to DNA fragmentation and germ cell damage in the testes, a finding that has been corroborated in other studies [6]. The higher level of nitrite in the testicular homogenates of BUS-treated rats can combine with toxic oxygen metabolites, including superoxide anion to form stronger oxidant molecules that causes lipid peroxidation, DNA damage, and oligospermia [51–53]. This has been found to be true in other models of testicular injuries because the testis is vulnerable to ROS-induced formation of MDA and together with apoptosis could lead to failure of spermatogenesis [54]. Therefore, the elevated testicular NO and declined ASCB concentrations and damage to the DNA in the present study are also accounted for the testicular oxidative damage and deficits of spermatogenesis promoted by BUS as reported in many studies [6, 8, 11, 12]. Our findings are in agreement with most of these studies.

Interestingly, ASCB cotreatment decreased superoxide dismutase activity and H2O2 concentration and increased catalase activity. Furthermore, lipid peroxidation, DNA fragmentation, and GSH level were also high in the BUS + ASCB animals, confirming the complementary effects of ASCB intake during BUS chemotherapy [26]. In addition, the elevated nitrite and diminished ASCB level are suggestive of the fact that vitamin C intake during BUS treatment could also increase the oxidative stress effect of BUS in the gonads. Because BUS action on the male gonads is a side effect of the drug, ASCB cotreatment does not protect the testes of rats against BUS-induced oxidative and DNA damage. Hence, the intake of ascorbic acid during BUS treatment increases the oxidative stress potency of the anticancer drug and does not preserve the testicular antioxidant machinery against BUS-induced oxidative damage. Whether the same scenario is applicable in the testis of cancer patients or in preclinical models remains unknown, but several studies have established that ascorbic acid acts as a supplemental pro-oxidative agent and enhances the actions of anticancer drugs in a synergic manner [20, 26]. This finding support the current study that ASCB increases the toxicity of BUS in the testis of a normal healthy rat model. The fact that ASCB

![Figure 7](image-url)
treatment could increase testicular ASCB which was diminished in the BUS + ASCB animals indicate that the ascorbic acid was greatly consumed during BUS-induced oxidant stress resulting in the increase of a collateral oxidative damage in the testis [20, 30, 55].

Because ASCB is relevant for testicular functions, the high H$_2$O$_2$ concentration in the ASCB animals is not surprising to us because increased superoxide dismutase activity would produce more testicular H$_2$O$_2$ that could contribute to the physiological effects of ASCB in the testes [56, 57]. In other models of testicular injury, ASCB cotreatment was found to counteract oxidative stress induced by pro-oxidants [50], a finding that is different from our current results. It appears that anticancer drugs administered to a normal healthy rat creates a local environment that is characterized by redox imbalance favorable for the pro-oxidant effect of ascorbic acid [26] and could be the basis for the synergistic effect of ASCB in the present study. To support this assumption, it has been shown that ASCB in combination treatments with anticancer drugs could mobilize Fe$^{3+}$ from tissues and convert to Fe$^{2+}$ allowing for the production of damaging oxygen radicals (Fenton reaction), with the capacity to induce oxidative damage [25, 26, 58]. Therefore, in studies concerning testicular oxidative stress, the noxious chemical inducing the effect in a non-target tissue plays important function in the antioxidant actions of the counter-acting agent. This information would be helpful at improving the understanding on how to develop effective combination treatment strategies against the collateral damage caused by anticancer agents or any other testicular toxins because it would determine if the combination treatment regimens would lead to synergism, amplified efficacy, and/or decreased toxicity [26].

Finally, the pattern of testicular morphologies in the BUS + ASCB animal, including the presence of single layers of germ cells on the basement membrane, decreased spermatogenesis score index, and absence of many germ cells in the seminiferous tubules, confirms the histological aberrancies of the testes and arrest of spermatogenesis in these group of animals and the inability of ASCB to protect spermatogenesis and the morphologies of the testes. Because this is the first time to report these findings, it is not known if similar biochemical effects and histological deficits can be seen in the testes of humans receiving vitamin C during chemotherapy treatment.

In conclusion, the intake of ascorbic acid during BUS treatment enhances the deregulation of the testis redox state, as shown by the low activity of superoxide dismutase, GSH and H$_2$O$_2$ concentrations, and higher activity of catalase and endogenous nitrite concentration resulting in the occurrence of collateral oxidative damage in the testis of a preclinical healthy rat model. This is interpreted to mean that, as the efficacy of BUS treatment is improved by ASCB cotreatment, a collateral damage on the testis would occur. Therefore, ASCB cannot be a drug of choice to protect the male gonads against chemotherapy-related side effects that are linked with the deregulation of the redox state of the testis.

**Data Availability**

Data supporting this research article are available from the corresponding author on reasonable request.

**Ethical Approval**

The study was approved by the Department of Biochemistry Ethical Committee for Research on Laboratory Animals (UPH/BCHR EC2022/011) and was in accordance with the Standard Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication Number, 85-23).

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors’ Contributions**

OEE: conceptualization and investigation; SOA: conceptualization, investigation, supervision, data curation, writing of draft manuscript, and approval for submission.

**References**


