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Effects of Licorice *(Glycyrrhiza glabra)* on testicular oxidative stress in sleep-deprived male Wistar rats

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Abstract

Background: Sleep deprivation is a public health problem that causes oxidative stress injury. Research evidence agrees that oxidative stress serves as an underlying factor in many chronic debilitating diseases. This study investigated the effect of aqueous Licorice extract (*Glycyrrhiza glabra*) a medicinal plant with known antioxidant activity on testicular oxidative stress parameters.

Methods: Twenty-five male Wistar rats were randomly allocated to and administered one of the following treatment regimens daily for five days: CONTROL – Distilled water, Sleep – Deprived (SD) – Distilled water, Sleep Deprived +Sleep Recovery (SD+SR) – Distilled Water, SD+LICORICE (SD+LICORICE) – 150?mg/kg bodyweight of Licorice and Sleep – Deprivation + Sleep-Recovery + Licorice (SD+SR+LICORICE) – 150?mg/kg bodyweight of Licorice. The rats in Sleep – Recovery groups were allowed to sleep in their cages after sleep deprivation protocol for 5 days each treatment regimen had *ad libitum* access to standard rat chow. After the experiment, the rats were sacrificed and blood was collected. Serum cortisol and testosterone were taken alongside testicular Glutathione, Catalase, and Malondialdehyde.

Results: Sleep deprivation significantly raised cortisol level and decreased testosterone levels both of which were reversed by Licorice administration. Significant reduction in Malondialdehyde (MDA) in rats treated with Licorice with a simultaneous increase in both GSH and CAT was also observed.

Conclusions: The antioxidant activity of licorice (Glycyrrhiza glabra) aqueous extract on the testis of rats exposed to oxidative stress suggests the potential of using this traditional medicinal plant in preventing oxidative injury caused by sleep deprivation.

Keywords: Antioxidant activity; Glycyrrhiza glabra; Oxidative stress injury; Sleep Deprivation.analytes, Sokoto

Introduction

Sleep deprivation is the failure to achieve optimal quality and duration of sleep either due to lifestyle, environmental or psychological issues, and unfortunately, sleep deprivation has attendant adverse health challenges that are often overlooked among scientists and shift workers such as doctors, nurses, and other healthcare workers (1). Furthermore, research evidence shows that sleep deprivation is associated with almost 50% of the top 15 leading causes of mortality in the United States (2). With the increase in industrialization, there is an increase in the use of technologically driven products and services therefore, workers have to keep up with the demands for such services by increasing work hours by staying awake for a longer time (3). Also,

the use of smartphones and other electronic media such as gaming gadgets keep people awake longer than planned particularly before bedtime could also be a contributory factor to the prevalence of sleep deprivation (4).

Sleep deprivation is reported to cause the production of excessive reactive oxygen species. This excessive reactive oxygen species diminishes the body's endogenous antioxidants in various tissues in the body: a state called oxidative stress. Oxidative stress describes the sequel of excessive production of reactive oxygen species for whatever reason at the expense of that which the endogenous antioxidant system can mop up leading to the destruction of various biomolecules in the body such as proteins, lipid, and nucleic acids (5). Oxidative stress is strongly linked to a myriad of pathological consequences such as the increased risk for diabetes mellitus, impaired cognition, and an increased risk for all-cause mortality in otherwise healthy individuals (6). Furthermore, oxidative stress also causes a de-synchronization of homeostatic mechanisms in the body, deregulation of the hypothalamic-pituitary-adrenal and gonadal axis, and impairment of the male reproductive system (7).

There has been a renewed interest in the disorders of the male reproductive system as much as interest in the use of medicinal plants for male reproductive system-related diseases (8,9). Besides, the World Health Organization posited that about 4/5 of the world population depends on the medicinal plant (roots, leaves, or extracts) of indigenous origin for health uses (10). Licorice, also known as *Glycyrrhiza glabra* is of modern science attention although its use dates back to pre-historic times. It is a natural sweetener, consumed as a beverage; it has several health benefits ranging from anti-diabetic, chemo-preventive, and antioxidant properties (11). The active ingredients in this medicinal plant have been well documented with Glycyrrhizin being its major active constituent (12). Although licorice has been well investigated, the potential health benefit of licorice administration on sleep deprivation particularly testicular oxidative stress has not been explored. Therefore, we aimed at testing the hypothesis that licorice could counteract the detrimental effects of sleep deprivation-induced oxidative stress on the testicular profile of rats. To achieve this, we examined the effect of aqueous extract of licorice on selected hormones, oxidative stress biomarkers.

Materials and Methods

Animals

Twenty-five male Wistar rats within the ages of five to six weeks, and weighing between 180-200g were bought from the animal house of the Ekiti State University, Ekiti-State, Nigeria, and used in this study. The rats were housed and maintained in standard conditions of light, feeding, and temperature in the Animal House. The study was carried out according to the ethical guidelines of the Ekiti State University ethical committee and the Guide for the Care and Use of Laboratory Animals (13). Rats had unrestricted access to standard rat chow and tap water. The experiment lasted twenty-four days. The first fourteen days were set aside for acclimatization to the laboratory environment, the animals were randomly assigned to one of the following experimental groups (n = 5 per group) and treated for five days for treatment groups, while sleep recovery groups had additional five days of sleep recovery accordingly.

Rats in Group I (Control) had 10ml/kg of distilled water orally, daily. Rats in Group II (Sleep-Deprived) designated SD had 10ml/kg of distilled water orally, daily. Those in Group III (Sleep-Deprived/Sleep-Recovery) designated SD + SR received 10ml/kg of distilled water orally, daily. Rats in Group IV (Sleep-Deprived with Licorice) designated as (SD + Licorice) received 150mg/kg bodyweight of Licorice orally, daily. Group V (Sleep-Deprived/ Sleep-Recovery with Licorice) designated (SD + SR + Licorice) received 150mg/kg bodyweight of Licorice orally, daily.

Extract preparation

Licorice root powder with batch no: LRP-2017/02 was sold by Herbs and Crops Overseas, India, and bought through Amazon. To prepare the extract, fifty grams of licorice powder was mixed with a 100ml of sterile water in a beaker with intermittent shaking for twenty minutes. The mixture was then filtered firstly through a muslin cloth for coarse residue, and through Whatman No.1 filter paper in an airtight coloured container. The extract was prepared and dosage fixed at 150mg/kg body weight according to a method earlier described by Fabunmi and colleagues (14).

Ethical clearance

All experimental procedures were approved by the host institution's Animal Ethics Screening Committee; clearance certificate number: EKSU/A67/2019/02/009

Sleep deprivation model

Twenty-five rats of weight 180-200g were used in the research. The rats were habituated for seven days under an ambient temperature of 25^{o}C and standard photoperiod of 12hr light-dark cycle after which they were subjected to Paradoxical sleep deprivation for 20hrs (11:00 am-7:00 am next morning) for 5 days with 4hr (7:00 am-11:00 am) rest daily using Modified Multiple Platform (MMP) method (15). Briefly, Large containers 1000 x 550 x 260 mm having seven small platforms (each 15cm in height) were used in this study for sleep deprivation. The large containers were filled with water up to 10 mm from the platform tops. Animals could move freely from one platform to the other but not sleep because the top of the small platforms was only wide enough for the animal to stand at alert. Once the animals sleep, their tail either touches the water and the animals are forced to wake up. Sleep deprivation was for 5 days and the tank cleaned and water changed daily throughout the SD period. The control group was allowed to sleep in their cages. In the sleep recovery model, the animals were given a sleep recovery period of 5 days after 5 days of sleep deprivation.

Determination of biochemical parameters

Rats were sacrificed in stages: Sleep-deprived groups at the end of five days sleep deprived and sleep recovery groups at the end of five days of sleep recovery. The rats were anesthetized with a mixture of 25% (w/v) urethane and 1% (w/v) alpha chloralose (5ml/kg; intraperitoneally., BDH Chemicals Ltd., Poole, England). The animals were humanely sacrificed and samples were collected via cardiac puncture with 5mls syringes after the opening of the upper abdominal region. Blood samples were collected into plain bottles and centrifuged at 3000r/p for 10 minutes. The serum samples were micro-pipetted into plain bottles and were immediately stored at -4^{0}c.

Testosterone estimation

Testosterone content was determined using an ELISA kit (Accubind, Monobind Inc. Lake Forest, USA). The microplate was washed four times and the substrate solution was added after incubated for one hour. Optical density was measured and the testosterone concentration was estimated at a wavelength of 620nm (16). This assay is based on the principle of competitive binding.

Cortisol estimation

This assay is based on the principle of competitive binding and the Cortisol content was determined using an ELISA kit (Accubind, Monobind Inc. Lake Forest, USA). The microplate was washed four times and the substrate solution was added after incubated for one hour. Optical density was measured at 450nm (17).

Homogenization of testicular tissue samples

Testicular tissue samples were quickly excised and subsequently washed in cooled 0.15M NaCl and then homogenized in 2ml of ice-cold buffer (potassium phosphate) (0.1M, pH: 7.4) using a makeshift homogenizer. Samples were centrifuged at 5000r/m for 15 minutes to get the

supernatant. The supernatant was micro pipetted into 3 different plain bottles and stored at -4^{0}c before the estimation of GSH (glutathione), CAT (catalase), and MDA (malondialdehyde).

Estimation of testicular glutathione

Testicular GSH was assayed as follows: The following was added to 0.1mL of the sample; 0.9mL sterile water and 1.5mL of precipitating reagent were added (3.34 grams of metaphosphoric acid, 0.4 grams of EDTA, and 60.0 grams of sodium chloride). The tubes were shaken together and left to stand for about 5min at room temperature (25) after they were centrifuged for 20min at 4000 rpm at 4 . 4.0mL of phosphate solution (0.3M disodium hydrogen phosphate) and 0.5mL 5-50-dithiobis-(2-nitrobenzoic acid) (DTNB) (80mg in 1% sodium citrate) were added to 1.0mL of the supernatant obtained. The formation of the yellow color complex was read immediately at 412 nm. A GSH standard curve was prepared and concentration was extrapolated and stated as mM of GSH/mg protein. (18).

Estimation of testicular catalase enzyme

This was done by UV spectrophotometric method that is based on monitoring the change of 240nm absorbance at high levels of hydrogen peroxide solution ($\geq$ 30*mM*). There is an inhibition of catalase enzyme whenever there is a high level of hydrogen peroxide. This leads to an alteration of the structure of the active site however there is a variation to the extent to which it occurs. Catalase was expressed in mmol/min/ml (16).

Estimation of testicular malondialdehyde

MDA assay is based on the degradation of polyunsaturated lipids and the substance gotten from the degradation is used to measure the level of lipid peroxidation. MDA reacts with thiobarbituric acid (TBA) as thiobarbituric acid reactive substances (TBARS) to form an MDA-TBA adduct, with absorbance at 532 nm. Concentration was subsequently stated as mmol/mg of protein (19).

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Statistical group analysis was performed with Graph pad (Prism 7) statistical software. Test of variance was done using One-way ANOVA and Tukey multiple comparisons test was done. Statistically significant differences were accepted at $p < 0.05$.

Results

Table 1 shows a significant increase (P<0.05) in the level of cortisol in SD when compared with CONTROL. There was however a significant reduction (P<0.05) in the cortisol level of SD+SR, SD+LICORICE, and SD+SR+LICORICE when compared to the Group SD. There was a significant reduction (P<0.05) in the level of testosterone in SD when compared to the CONTROL while SD+SR, SD+LICORICE, both showed a significant increase in testosterone level (P<0.05) compared to SD, unlike SD+LICORICE which was not significant.

Table 1: Effects of aqueous licorice extract on selected hormones in normal and sleep-deprived/ recovery male Wistar rats

	Control	SD	SD+SR	SD+licorice	SD + SR+licorice
Cortisol(ng/ml)	30.97±1.769	72.09±3.151^a	42.47±1.773^b	38.40±3.311^b	24.01±2.846^b
Testosterone (ng/ml)	1.776±0.092	1.147±0.044^a	1.673±0.117^b	1.317±0.133	1.776±0.065^b

Data expressed as means ± SEM, n = 5. One-way ANOVA.

Key: Sleep deprivation (SD); Sleep deprivation and Sleep recovery (SD+SR).

The result in Figure 1 shows a significant increase in the GSH level (P<0.05) in the SD group when compared to the CONTROL group. There is a significant reduction in GSH levels (P<0.05) in SD+SR, SD+LICORICE, SD+SR+LICORICE, compared to the SD group.

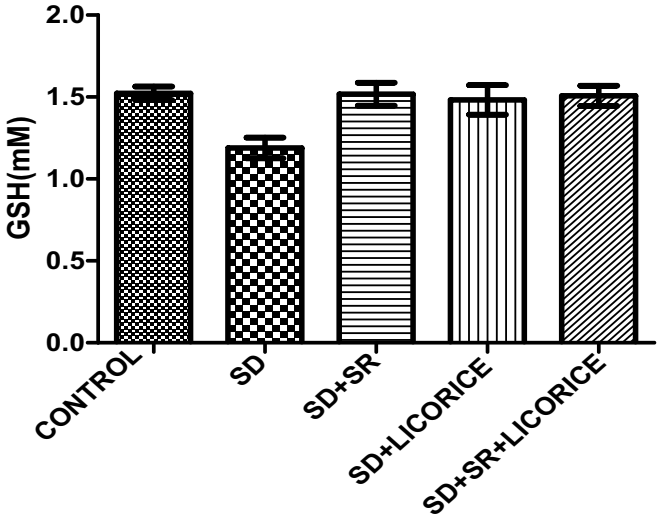

Figure 1: Effects of aqueous licorice extract on serum glutathione level in normal and sleep-deprived/ recovery male Wistar rats.

Data were analysed using one-way ANOVA and Tukey multiple comparisons test was done using Graph pad (Prism 7) statistical software. Data are expressed as means ± standard error of the mean (SEM) n = 5. Statistically significant differences were accepted at $p < 0.05$.

Key: Sleep deprivation (SD); Sleep deprivation and Sleep recovery (SD+SR)

Results in figure 2 show a significant reduction in the serum concentration of the catalase level ($P<0.05$) in the SD group when compared to the CONTROL group. There was a significant increase in serum catalase level ($P<0.05$) in SD+SR, SD+LICORICE, SD+SR+LICORICE compared to the SD group.

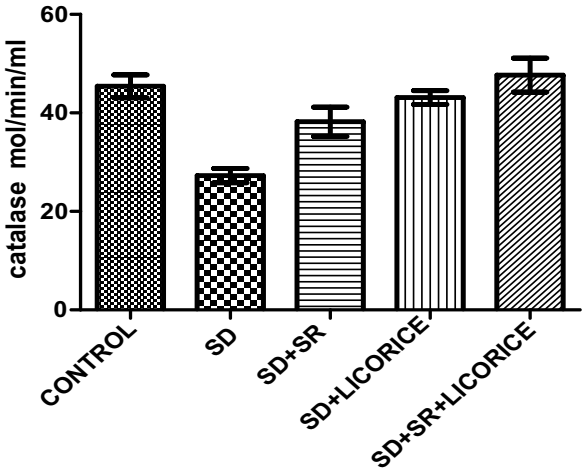

Figure 2: Effects of aqueous licorice extract on Catalase level in normal and sleep-deprived/ recovery male Wistar rats.

Data were analysed using one-way ANOVA and Tukey multiple comparisons test was done using Graph pad (Prism 7) statistical software. Data are expressed as means ± standard error of the mean (SEM) n = 5. Statistically significant differences were accepted at $p < 0.05$.

Key: Sleep deprivation (SD); Sleep deprivation and Sleep recovery (SD+SR)

Results in Figure 3 show a significant increase in the serum concentration level of MDA ($P<0.05$) in the SD group when compared to the CONTROL group. There was a significant reduction in serum MDA levels ($P<0.05$) in SD+SR, SD+LICORICE, SD+SR+LICORICE, compared to the SD group.

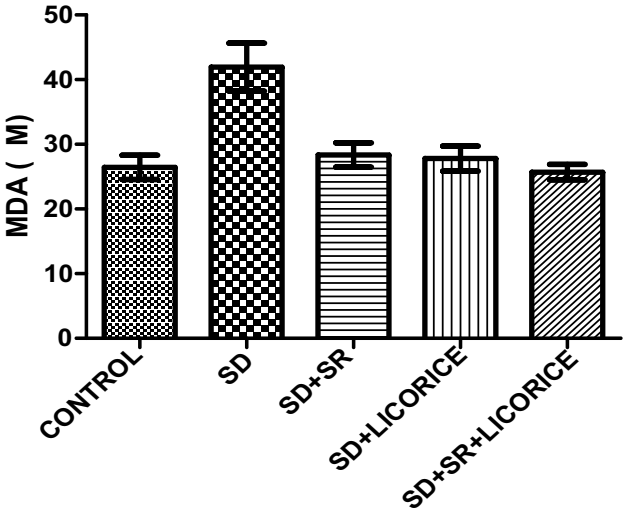

Figure 3: Effects of aqueous licorice extract on Malondialdehyde level in normal and sleep-deprived/ recovery male Wistar rats.

Data were analysed using one-way ANOVA and Tukey multiple comparisons test was done using Graph pad (Prism 7) statistical software. Data are expressed as means ± standard error of the mean (SEM) n = 5. Statistically significant differences were accepted at $p < 0.05$.

Key: Sleep deprivation (SD); Sleep deprivation and Sleep recovery (SD+SR)

Discussion

This study revealed that the aqueous extract of licorice ameliorated sleep deprivation-induced testicular oxidative stress by augmenting endogenous testicular antioxidant status while simultaneously scavenging excess free radicals.

Sleep-deprived rats exhibited signs of chronic stress by having raised cortisol level as against significantly decreased testosterone level a scenario earlier described by Ajibare et al (20) as probably a cause and effect relationship where decreased testosterone production is secondary to elevated cortisol level. The latter causing programmed cell death in the testosterone-producing cells of Leydig. Also, (21) also supported this same relationship between cortisol and testosterone in the setting of chronic stress while positing that low testosterone is key in physical and psychological stress response in males.

While it is true that licorice (in large quantities and prolonged use), might have some deleterious effect on the aldosterone system (22). It could be argued that licorice still possesses beneficial effects in some regard because studies have shown that aqueous licorice extract administration resulted in dose-dependent increases in plasma renin and sodium with a simultaneous decrease in plasma cortisol and aldosterone (11). This position is in concord with our study where licorice reduced elevated cortisol level seen after sleep deprivation. It is plausible that this action is due to the presence of glycyrrhizin and glycyrrhizic acid which are known to be inhibitors of 11β-hydroxysteroid dehydrogenase with isoforms 1 and 2 (11β-HSD1 and 11β-HSD2). In vivo cortisol is modulated by the former (11β-HSD1) which converts inactive glucocorticoids to active cortisol. Glycyrrhizic acid inhibits 11β-HSD1 more thereby leading to the production of inactive glucocorticoids (23).

Licorice has a corticosteroid-like chemical structure and as the daily administration/ consumption goes on, it initiates some endocrine changes such as blocking the 17, 20 lyase, and 17-hydroxy-steroid dehydrogenase enzymes. This initially manifests as a reduction in the serum testosterone. However, the negative feedback mechanism is initiated thereby activating the hypothalamic-pituitary-gonadal axis to produce luteinizing hormone. These over-rides the enzymatic block ultimately increase testosterone levels after some days (24) This explains why there was no significant change in testosterone levels after 5 days in the SD+LICORICE group as supported by the work done by Salem and colleagues on mice (25). On the other hand, during sleep recovery, there is a restoration of balance in the hypothalamic-pituitary-gonadal axis which ultimately allow for the restoration of testosterone level, besides, slow-wave sleep boosts testosterone level (20) along with the fact that the binding of licorice to 17, 20 lyase and 17 hydroxysteroid dehydrogenase hormone is weak and transient, there is an increase in testosterone in the SD+SR+LICORICE animals.

During sleep deprivation, there is an increase in the rate of neuronal metabolism which increases the oxygen demand in the brain. As the body tries to increase oxygen generation to meet up with the demand, about 1% of the generated oxygen is transformed into highly reactive free radicals (which are pro-oxidants) these free radicals overwhelm the endogenous antioxidant system and damages cell membrane in tissues(26). Oxidative stress occurs when there is an excess of pro-oxidant species (reactive oxygen species) compared to antioxidant species with attendant damage which could either be at the cellular level, molecular level, or both (27). As a principle, during oxidative stress, the first line of defense of call is the endogenous antioxidant system such as reduced glutathione (GSH), Catalase (CAT) These endogenous antioxidant systems are overwhelmed and are drastically reduced while

reactive oxygen species are being produced concomitantly such as seen when lipid peroxidation biomarkers such as Malondialdehyde (MDA) is elevated. All these findings were consistent with ours and are supported by other previous studies (28,29) thus strengthening the link between sleep deprivation and oxidative stress parameters. Of note, however, is the peculiarly high susceptibility of the testis to oxidative stress due to the abundance of polyunsaturated fats (30).

Licorice administration offers protective effects from sleep deprivation-induced testicular oxidative stress. *In vitro* and *in vivo* studies demonstrated that the antioxidant ability of licorice is due to the presence of phenolic compounds and flavonoids that exert oxygen scavenging, metal ion chelating, hydrogen-donating abilities, and reducing activities (31). In this study, rats treated with the aqueous licorice extract had an elevated level of GSH. It is plausible that glycyrrhizin present in licorice counteracted the oxidative stress in these rats by effectually scavenging and interfering with the conjugation of reactive oxygen species to GSH, as evident from restored GSH content. This agrees with the studies done on mice (32), the brain of sleep-deprived rats treated with licorice, (14), and the liver of rats with CCL4 induced oxidative stress (33) in the. Furthermore, aqueous licorice extract restored the testicular catalase in sleep-deprived rats according to our study. This is in concord with earlier studies done by Sakr and Shalaby (34) in carbendazim induced testicular oxidative stress and in the liver of sleep-deprived rats (35). Concomitantly, malondialdehyde levels were also reduced in the sleep-deprived rats treated with licorice plausibly because it has strong anti-lipid peroxidation activity possessed by isoliquiritigenin has posited by Lee *et al* (36) where it restored tissue catalase activity in cisplatin-induced oxidative stress. Likewise, this Sleep recovery restored the antioxidant level of both CAT and GSH while simultaneously reducing the marker of lipid peroxidation (MDA) probably because of the antioxidant boosting effect of sleep which gave room for scavenging of free radicals (37).

Conclusion and limitations

Overall, these findings assert that licorice mitigated sleep-deprivation induced oxidative stress in male rats and this ability maybe due to the presence of triterpene saponins and many phenolic compounds present in it. The authors acknowledge the absence of another normal group that could have been treated with licorice and be beneficial in comparison with the other treatment groups. Also, biomarkers of specific brain tissue that control the physiology of sleep were not included in this study.

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