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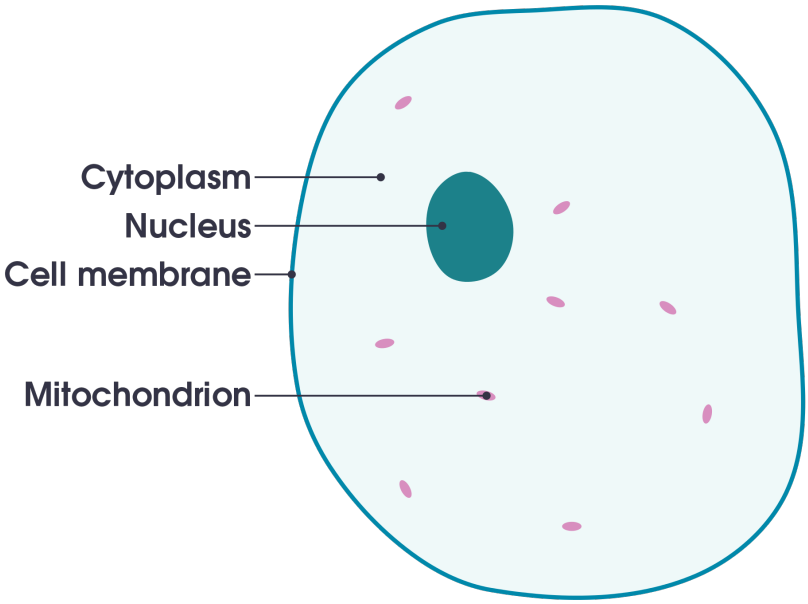

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Short Communication

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Original Research

The potential role of mesenchymal stem cells in a hypoxia model induced by sodium nitrite in testes of male albino rats

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Abstract

Introduction: The present work aims to examine the possible role of stem cells on biochemical markers and histopathological alterations of hypoxia caused by sodium nitrite ($NaNO_2$) toxicity in testes of male rats. **Methods**: In this study, 96 adult male albino rats were divided into 6 groups (16 rats each). Group 1 (G1) was the control group and received distilled H_2O. Group 2 (G2) received daily $NaNO_2$ (35 m/kg bwt/ day) via subcutaneous injection for 3 weeks. Group 3 (G3) received $NaNO_2$ for 2 weeks and were then injected once with $2*10^6$ mesenchymal stem cells (MSCs) intravenously and sacrificed 4 weeks later. Group 4 (G4) received $NaNO_2$ for 2 weeks and were then injected with $2*10^6$ MSCs followed by daily $NaNO_2$ injection for 1 week; rats in G4 were sacrificed 4 weeks from MSCs treatment. Group 5 (G5) rats were treated with $NaNO_2$ for 2 weeks and then left to recover for 4 weeks. Finally, Group 6 (G6) rats were treated with $NaNO_2$ for 3 weeks and left to recover for 3 weeks, after which point they were sacrificed. **Results**: The results showed that $NaNO_2$ caused oxidative damage and histopathological alterations in the rat testes, as well as increased the levels of testes malondialdehyde (MDA), nitric oxide (NO) and DNA fragmentation percentage (DNA F %). Moreover, $NaNO_2$ decreased the elevated activities of testes catalase (CAT) and total antioxidant activity (TAA), in comparison to the control group. The histological results illustrated different distortions, vacuolization and lipid accumulations in interlobular space as well as diminution of inter cellular germ cell layers, absence of Leydig cells, irregular basement membrane of tubule, and separation within spermatogenic cells. In addition, congestion and dilation of intertubular and peripheral blood capillaries were found. Nevertheless, the administration of stem cells reduced the danger actions of sodium nitrite by enhancing biochemical marker concentration.

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Conclusion: There was an improvement in the histology of the rat testes, including a relatively normal order in the different stages of spermatogonia and loss of different stages of spermatocytes. Regarding the recovery period, there was also a significant improvement in each of the biochemical parameters assessed and in the histological lesions.

Keywords

Sodium nitrite, hypoxia, stem cell, testes oxidative stress, testes histology

Introduction

Sodium nitrite ($NaNO_2$) is a familiar preservative for meat and dye with both dangerous and beneficial effects. Processed meats, such as hot dogs, bacon, sliced deli meats and sausage, contain $NaNO_2$ in its list constituents. Sodium nitrite is utilized occasionally for medicinal purposes and may be employed as a component in the treatment of sickle-cell anemia and heart attack (Cosby et al., 2003; Mack et al., 2008). It is a water soluble and inorganic salt extensively used in different industries, including the agricultural, textile processing, chemical, coloring, and disinfectant industries (U.S.DHHS, 2001).

Hypoxia refers to low oxygen concentration in the body or organs. The condition begins from an inequality between the quantity of oxygen needed through the body and the quantity of oxygenated blood that is provided (Maher et al., 2008). Numerous studies have shown that the harmful impact of $NaNO_2$ induced hypoxia is related to inflammation, oxidative stress and methemoglobinemia, which induce injury and dysfunction of different organs (Al-Gayyar et al., 2014; El-Sheikh and Khalil, 2011; Hassan et al., 2009; Salama et al., 2013).

The body responds to hypoxia by adaptive reactions, such as angiogenesis, smooth muscle relaxation and vasodilatation, thereby elevating tissue blood supply and compensating for the lack of oxygen. The hypoxia-driven adaptive reactions also cause an elevation in the temperature of the testes (Farias et al., 2005). The rise in temperature activates apoptosis in cells of the seminiferous tubules of the testis. The exposure to continuous and intermittent hypoxia changes the male mouse reproductive system. Greater testicular damage have been observed under continuous hypoxia. With intermittent hypoxia, lesions are less essential, suggesting that cycles of normoxia compensate the effects on the testicle (Vargas et al., 2011).

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Stem cells are able to regenerate and are capable of differentiating into specialized cell types (Bruder et al., 1997; Chopp et al., 2000; El Asmar et al., 2011; Sato et al., 2005). Anderson *et al*. (2001) reported that stem cells have the potential to treat various diseases, inducing diabetes, liver, heart illness and infertility. Adult mesenchymal stem cells (MSCs) are believed to be highly promising applicant cells for renewal applications for the reason that they have a high proliferative capability and the ability to differentiate into specified cells (Caterson et al., 2002; Nöth et al., 2002). In fact, MSCs are self-renewing cells which are able to differentiate into several mesodermal tissues, e.g. bone, fat, cartilage and muscle (Pittenger et al., 1999), and to migrate toward damaged tissue sites (Fujimoto et al., 2012). The present work aims to evaluate the action of $NaNO_2$ induced hypoxia on male rat testes and the therapeutic role MSCs play in the deleterious effects of $NaNO_2$ on testes.

Materials - Methods

Experimental animals

In the present study 96 adult male albino rats (weighing 150-180 g) were used. Rats were obtained from Ain Shams Hospital Animal House. Rats were left for two weeks for acclimatization before starting the experiments. Animal procedures and experimental protocols were approved by Ain Shams University authorities; they were in accordance with the Egyptian animal protection rules and consistent with the guidelines of the European Communities (EC) (1986).

Sodium nitrite ($NaNO_2$) administration

$NaNO_2$ (7632-00-0) was obtained from Alahram Company (Al-Sadat city, Egypt) and dissolved in distilled water at 27^0C. It was administered subcutaneously to rats at a dose of 35 mg/kg bwt/day, as previously described by Bhanumathy *et al*. (2010) (Bhanumathy et al., 2010).

Isolation, propagation, identification and labeling of MSCs derived from bone marrow of rats

Bone marrow was removed from the tibiae and femurs of male rats, aged 6 weeks old, and flushed in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) enhanced with 10% fetal bovine serum (FBS, GIBCO/BRL). The nucleated cells were then separated in a density gradient via Ficoll/Paque (Pharmacia) and re-suspended in whole culture medium complemented with 1% penicillin streptomycin (GIBCO/BRL). Cells were cultured at 37°C, 5% humidified CO_2 for 12-14 days during the primary culture. The medium were replaced each 2-3 days.

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When colonies grew large (80-90% confluence), the culture was rinsed with PBS twice and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were re-suspended with serum-supplemented culture and kept warm in 50 cm^2 culture flasks (Falcon). The resultant media were considered first route supernatant cultures (Alhadlaq and Mao, 2004). On day 14, the adherent cell colonies were trypsinized and counted.

Cells were recognized as MSCs by the identification of their morphology, adherence, and ability to transform into osteocytes (Jaiswal et al., 1997) and chondrocytes (Seo et al., 2009). Transformation into osteocytes was attained by adding 1 to 1000 nM dexamethasone, 0.25 mM ascorbic acid, and 1 to 10 mM beta-glycerophosphate to the culture. Transformation of MSCs to osteoblasts was attained through morphological changes and Alzarin red staining of differentiated osteoblasts. Transformation to chondrocytes was attained by adding 500 ng/ml bone morphogenetic protein-2 (BMP-2; R&D Systems) and 10 ng/ml transforming growth factor-β3 (TGF- β3) (Peprotech, London) for 3 weeks (Seo et al., 2009). In vitro transformation into chondrocytes was verified by morphological changes and Alcian blue staining of differentiated chondrocytes. Flow cytometric analysis of cultured MSCs surface markers was also done.

In the current study, MSCs were tagged with PKH26 (Sigma, Saint Louis, MO), consistent with the manufacturer's advice. Cells were intravenously injected once into the rat tail vein at a dose of $2*10^6$ cells, according to previously published by Kebriaei et al. (2009) (Kebriaei et al., 2009).

Experimental Design

Rats were randomly split into six main groups with 16 rats each. Group 1 (G1) received distilled water and served as the control. Group 2 (G2) received daily subcutaneous (s.c.) $NaNO_2$ injections at a dose of 35 mg/kg bwt/ day for 3 weeks. Group 3 (G3) received $NaNO_2$ for 2 weeks and were then injected once with MSCs ($2*10^6$ cells) intravenously; rat were sacrificed 4 weeks after MSC injection. Group 4 (G4) were treated with $NaNO_2$ for 2 weeks and then treated with MSCs at 1 week following $NaNO_2$ injection; rats were sacrificed 4 weeks from MSC injection. Group 5 (G5) were treated with $NaNO_2$ for 2 weeks and then left to recover for 4 weeks and then sacrificed. Lastly, Group 6 (G6) received $NaNO_2$ for 3 weeks then left to recover for 3 weeks and then sacrificed.

Biochemical Studies

Testes samples were prepared by taking a weighted part of the testes and homogenizing in the recommended saline. Biochemical analyses were conducted, which included colorimetric determination of NO (carried out according to Miranda *et al.* (2001) (Miranda et al., 2001) using modified Griss reagent), measurement of malondialdehyde in the tissue (using a modified thiobarbituric acid (TBA) assay consistent with Draper and Hadley (1990) (Draper and Hadley, 1990)), percentage of DNA fragmentation (as determined by

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quantitative analysis using diphenylamine assay, according to the method described by Sharawy (2013) (Sharawy, 2013), and catalase activity determination (through a method used by Bock and Pavelka (1980) (Bock et al., 1980)). Moreover, total antioxidant activity was verified, according to Koracevic *et al.* (2001) (Koracevic et al., 2001).

Histopathological Examination

In the present study, testes specimens were carefully dissected and fixed in Bouin's solution for routine histological analysis, and implanted in paraffin wax at 60°C. Serial transverse sections were then cut at 5-6 microns in thickness using Cambridge Rocking Microtome and affixed to slides. For general histological examination, sections were stained with Hematoxylin and Eosin (Drury and Wallington, 1980).

Statistical Analysis

Reported data represented the mean ± SE for 8 animals per group. For statistical analysis, one-way analysis of variance (ANOVA) and post-HOC test ("least significant difference (LSD) analysis) were completed using statistical package for social science (SPSS) for Windows software (version 17). Statistical significance was set at $p<0.05$.

Results

Biochemical investigation

The results of the biochemical parameters are shown in **Table 1**. Oxidative stress parameters showed a significant increase in all treated groups with regards to the following: the value of testes nitric oxide (NO) contents, malondialdehyde (MDA) contents, and DNA fragmentation percentage (DNA F%). On the other hand, there was a significant reduction in catalase activity (CAT) and total antioxidant activity (TAA) of G2 group as compared to the G1 control group. Only G3 rats showed a non-significant change in MDA contents.

Moreover, there was a significant reduction in the recovery groups and the MSC treated groups with respect to testes NO contents, MDA contents, and DNA F%, when compared with those rats in G2 group. Conversely, testes catalase activities (CAT) and total antioxidant activities (TAA) were significantly increased in stem cell groups and recovery groups, compared to G2 rats.

Histological investigations

In the present study, sections from the testes of the control rats (G1) showed normal structures of seminiferous tubules and interstitial tissue (**Fig. 1A**).

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Spermatogonia was normal too, with primary and secondary spermatocytes/ spermatids developing as standard, and moving towards Sertoli cells (Fig. 1B).

Table 1. Total nitric oxide (NO) contents in the testes (mM/g), malondialdehyde (MDA) contents (uM/g), DNA fragmentation percentage (DNA F %), catalase activities (CAT) (u/g/sec), and total antioxidant activities (TAA) (mM/g/min) are shown for the various treated groups

Parameters Groups	NO	MDA	DNA F%	CAT	TAA
G1	64.96±0.811	28.91±1.189	33.68±0.669	0.32±0.012	46.32±0.357
G2	154.07±2.286^{a}	51.02±2.483^{a}	65.48±1.505^{a}	0.14±0.015^{a}	14.33±0.782^{a}
G3	79.71±1.254^{ab}	31.37±1. 575^{b}	38.62±1.760^{ab}	0.29±0.017^{b}	44.39±0.391^{b}
G4	107.75±1.019^{abc}	38.34±0.607^{abc}	47.28±0.459^{abc}	0.27±0.015^{ab}	31.32±0.521^{abc}
G5	112.45±2.250^{abcd}	42.18±2.057^{abc}	56.24±1.537^{abcd}	0.22±0.015^{abcd}	29.67±1.034^{abcd}
G6	139.72±0.938^{abcde}	48.55±0.781^{acde}	59.32±0.921^{acde}	0.18±0.038^{abcde}	22.67±0.450^{abcde}

Values are means of 8 rats ± SE at $p<0.05$. 'a' represents significant change from control group (G1), 'b' represents significant change from hypoxic (G2) group, 'c' represents significant change from G3 group, 'd' represents significant change from G4 group, and 'e' represents significant change from group treated with sodium nitrite for 2 weeks then left for recovery period (G5).

The hypoxic group (G2) showed seriously injured seminiferous tubules. Microscopic alterations were regarded as characteristic indications for seriously injured tubules. The greater part of the seminiferous tubules showed vacuolization, lipid accumulation in intertubular space with gelatinous material in intertubular connective tissues, and diminution of Leydig cells. Also, germ cell layers were reduced **(Fig. 1C).** Abnormally shaped seminiferous tubules manifested irregular tubule basement membranes within necrotic areas of the spermatogenic cells layers **(Fig. 1D).** Meanwhile, some seminiferous tubules appeared with cellular debris in the tubular lumen **(Fig. 1E).** Numerous giant cells were observed with cluster multinucleated nesting arrangement, karyorrhexis of Leydig cells, depletion of spermatocytes, hyaline material with absence of Leydig cells in interstitial connective tissues, and separated seminiferous tubule basement membranes.

Specifically, the G3 group revealed significant improvement of basement membrane and seminiferous tubules borders; some elongated tubules as shown in Fig. 1F**.** Moreover, the seminiferous tubules showed a nearly normal pattern of all the spermatogenic stages, activated Sertoli cells, and a slightly disordered and thickened outer basal layer.

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There were definite improvements of histopathological lesions seen after MSC therapy. These are clearly demonstrated in **Fig. 2A**. The testicular structure sections from G4 rats showed a relatively normal pattern of different seminiferous tubules, accompanied by an intertubular space (with dilated and congested interstitial blood vessels), hyaline substances, and a rare disturbance pattern of spermatocytes (**Figs. 2B,C**).

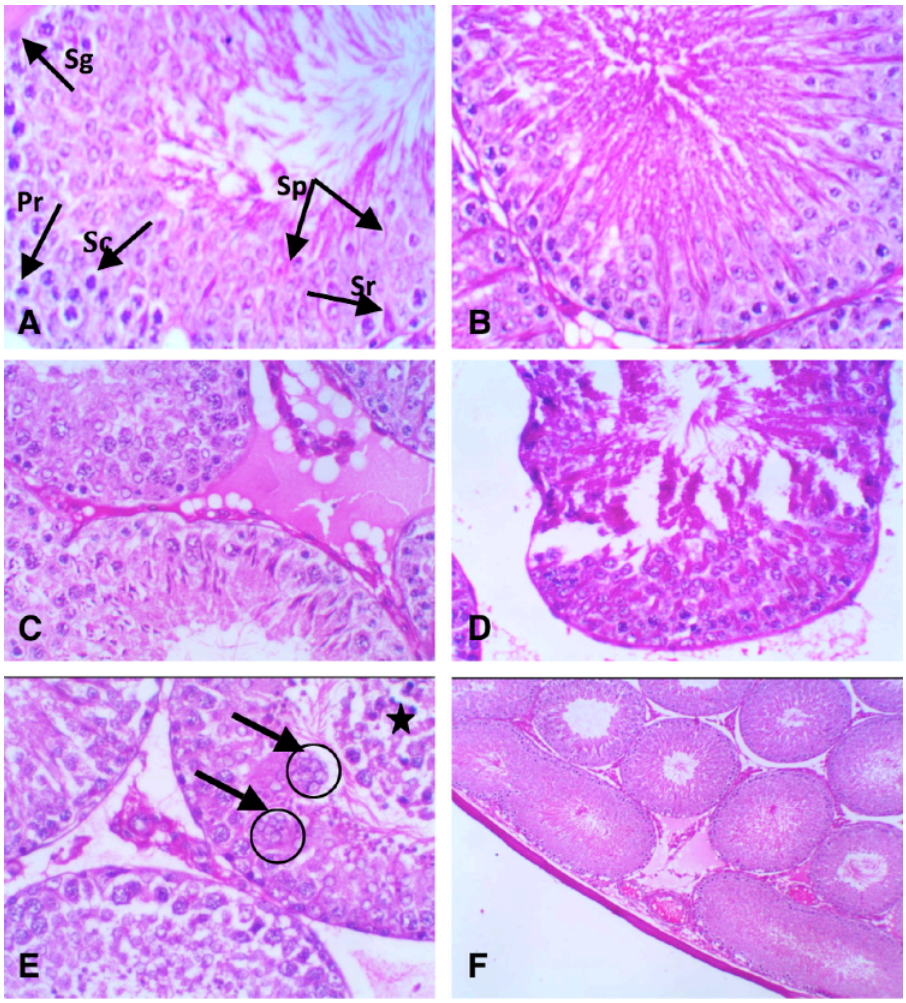

Figure 1. Histological organization of the seminiferous tubules of rats in the groups. A-B: (**G1 group**) demonstrates two microscopic images of rat testes: low magnification image of one seminiferous tubule and seminiferous epithelium including Sertoli (sr) and germ cells (sg) at different phases of primary spermatozoa (pr) ,secondary spermatozoa (sc) and spermatides (sp)**. C-E: (G2 group). Figure C** shows vacuolization and lipid accumulations in interlobular space with gelatinous material, and diminution of intercellular germ cell layers and absence of Leydig cells**. Figure D** shows disorganization of the seminiferous epithelium, as observed by degeneration within the seminiferous lumen filled with cellular debris, an irregular tubule basement membrane, and separation within spermatogenic cells**. Figure E** illustrates cellular debris in the tubular

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lumen, numerous giant cells with cluster multinucleated nesting arrangement (O) and Karyorrhexis of Leydig cells (arrow). **F. (G3 group)** showing a definite amelioration of basement membrane (normal appearance) and seminiferous tubules borders, with some other elongated tubules. (A-E: X400; F: X100).

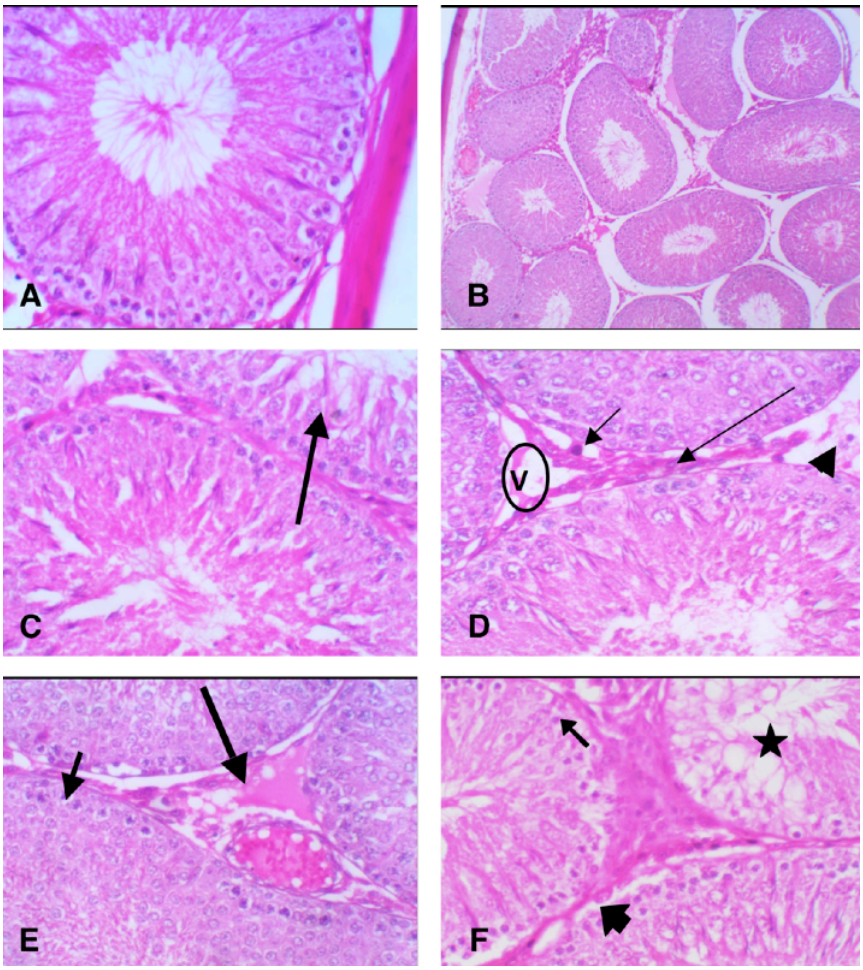

Figure 2. Testes sections from rat groups. A: Testes section from G3 group demonstrate the seminiferous tubules showing a nearly normal appearance with all spermatogenic stages, activated Sertoli cells, and slightly disordered and thickened outer basal layer (H&E X400). **B:** Testes section from G4 group showing a relatively normal pattern of different seminiferous tubules wading in the intertubular space with hyaline substances (H&E X100). **C:** Testes section from G4 rats showing a relatively normal order in the different stages of spermatogonia, and a rare disturbance pattern of spermatocytes (H&E X400). **D:** Testes section from G5 group showing the seminiferous tubules to be nearly normal with distinct pyknotic structures (short) with degenerative areas (head), but with Leydig cells still present (long) (H&E X400). **E:** Testes section from G5 showing a slight improvement in the testicular structures, an abnormal elongated shape of the seminiferous tubules, with slightly dilated and congested of interstitial blood vessel Leydig cells, and with hyaline substances in the background (H&E X400). **F:** Testes section from G6 group showing partially

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ameliorative seminiferous tubules with hyaline material, and an absence of Leydig cells in interstitial spaces, as well as evident erosion and hydropic degeneration (star), (H&E X 400).

The histological structure of sections of the testes of G5 rats (**Figs. 2D,E**) illustrated a slight improvement of the testicular structures. They appeared nearly normal, and the seminiferous tubules showed only a few abnormally elongated shapes. The interstitial blood vessels were slightly dilated and congested. There were also Leydig cells with hyaline substances and some degenerative patches in the intertubular spaces. The testes of G6 rats showed a partial amelioration of seminiferous tubules with hyaline material, an absence of Leydig cells in the interstitial spaces, and evident erosion and hydropic degeneration **(Fig. 2F).** Recovery of treated G5 rats revealed little pathological change, compared to G6 treated rats.

Discussion

Sodium nitrite is known as an inorganic salt which have a variety of industrial purposes. The adverse health effects of $NaNO_2$ in animals and humans typically lead to the formation of methemoglobin in the blood. This is due to cyanosis which can, at extremely elevated levels, lead to death. People are regularly subjected to sodium nitrite during nutrition and water (Pavlova et al., 2013). Hypoxia is recognized to obstruct fertility in man (Okumura et al., 2003) and in other mammals. Hypoxia can influence the function of the testes by diminishing the level of testosterone and by disturbing spermatogenesis (Farías et al., 2008).

In present study, $NaNO_2$ (at a dose of 35m/kg s.c.) had pathological effects of on the testes of rats. It led to a rise in MDA levels, as compared to control rats (which did not receive $NaNO_2$ daily injections). In accordance with our observation, Vossen and De Smet (2015) (Vossen and De Smet, 2015) have shown that antioxidants can be used as therapy to decrease nitrite toxicity (Al-Gayyar et al., 2014; Al-Gayyar et al., 2016). Interestingly, NO has been connected to an elevation in lipid peroxidation and MDA in both humans (Romeo et al., 2003) and experimental rats (Özdamar et al., 2004). A rise in NO following eNOS overexpression has been connected to germ cell apoptosis in a cryptorchidism germ mouse model (i.e. the absence of one or both testes from the scrotum) (Ishikawa et al., 2005).

The results obtained from the present work show that hypoxia caused an increase in testes DNA fragmentation percentage after administration of $NaNO_2$. This is compatible with Knaapen *et al.* (2005) (Knaapen et al., 2005) who recorded a rise in DNA fragmentation in adult rats and concluded that $NaNO_2$ induced DNA fragmentation due to oxidative stress. The rigid wrapping of chromatin, which depends on condensation and replacement of histones with

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protamines, is particularly essential as adult spermatocytes are not capable of fixing DNA damage (De Ambrogi et al., 2006; Laberge and Boissonneault, 2005). Free radicals possess the capability to precisely injure sperm DNA by assaulting the purine and pyrimidine units and the backbone deoxyribose. Generally, the DNA of sperm is firmly enveloped by protamines shielding it from the assault of free radicals. But, infertile men frequently show a lack of protamination, placing the DNA of the sperm susceptible to ROS assault (Oliva, 2006).

On the other hand, free radicals are able to initiate apoptosis in the sperm, causing caspase-mediated enzymatic dilapidation of the sperm DNA (Duru et al., 2000; Moustafa et al., 2004; Villegas et al., 2005; Wang et al., 2003). ROS may cause varying degrees of sperm dysfunction, depending on the amount of oxidative stress. Damage from ROS occurs primarily through two routes. First, ROS may be responsible for DNA fragmentation which is usually seen in the spermatozoa of infertile men by causing single- and double-stranded DNA fractures (Kodama et al., 1996). Second, higher levels of ROS also may cause damage through a chain of chemical reactions that result in lipid peroxidation of the sperm plasma membrane (Alvarez et al., 1987).

The present study clearly demonstrates that there is a considerable rise in DNA fragmentation in testes of $NaNO_2$-treated rats in comparison with control rats. David and Grongnet (2000) showed similar results; they reported that treatment with $NaNO_2$ led to increasing fragmentation (David and Grongnet, 2000). More extensive fragmentation of DNA was observed when the time of hypoxia exposure was increased.

In our study, treatment with MSCs may cause MSC differentiation into germ cells in the testis (Nayernia et al., 2006). The generation of oxidative stress in infertile rats is reflected by increased production of TBARS, a measure of lipid peroxidation in the homogenates of testes, according to Hussein *et al.* (2015) (Hussein et al., 2015), and which may explain our findings. In the cell membranes, polyunsaturated fatty acid residues react with oxygen radicals normally causing collection of lipid peroxidation yields, several of which also injure proteins and DNA. Leydig cells contain higher membrane lipid matter and may affect the vulnerability of the testes for lipid peroxidation in cadmium treated mice (Dobretsov et al., 1977; Georgiou et al., 1987; Hall, 1994).

Results of our study indicated a reduction in the testes CAT and TAA activities in hypoxic rats compared to control ones. Treatment with MSCs significantly restored those levels. CAT and TAA activities significantly increased in MSC treated rats whereas the $NaNO_2$ group showed a significant decrease in activities compared to the controls. These results could be explained by the inhibition of CAT activity. Titov and Petrenko (2002) suggested that the supraphysiological doses of NaNO2 inhibited catalase activity (Titov and Petrenko, 2003). This mechanism could clarify the initial observations that $NaNO_2$ defends H_2O_2 against damage by catalase activity in hemolysates.

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Moreover, applications of 50 – 100 μM $NaNO_2$ hinder myeloperoxidase enzymatic activity, stopping its utilization of H_2O_2 (Knaapen et al., 2005). Treatment with MSCs in the hypoxic groups led to a significant decrease of NO and MDA contents, compared to the control group. Also, CAT and TAA activities were increased significantly. In support of these results, El-Far *et al.* (2012) demonstrated that injection of MSCs led to a correction and fix of the inequity between ROS and antioxidant protection, thereby enhancing antioxidant-mediated defense and adjusting lipid peroxidation (Mohamed et al., 2012).

In the present study, the MSC treated groups were associated with a significant elevation in CAT and TAA activities, compared to G2 and other recovery groups. However, administration of MSCs significantly prohibited the impact of $NaNO_2$ on antioxidative regularity; it reduced NO and MDA and, in parallel, elevated the CAT activities in the tissue of the testes. These data are in agreement with Hussein *et al.* (2015) who demonstrated the antiperoxidative function of MSCs (Hussein et al., 2015). The defense mechanism by bone marrow MSCs, in vivo, against oxidative alteration may be due to its free radical hunting potential. Moreover, the precise reactions of MSCs to oxidative damage may play a vital role in control of tissue homeostasis in addition to renewal of organs following oxidative damage (Burova et al., 2013; Hassan and Alam, 2014).

Histological changes evoked in the testes by $NaNO_2$ administration were mainly alterations of the seminiferous tubules, including vacuolization, lipid accumulation in intertubular space (with gelatinous material in intertubular connective tissues), and diminution of Leydig cells. Other observed histological changes were reduced germ cell layers, and appearance of hyaline materiel in the lumen of seminiferous tubules and interstitial connective tissues. The results from our study are in accordance with the majority of other works that have revealed changes in the human male constituents of fertility by hypoxia, including reduction in sperm count, reduction in sperm mobility and alleviation in testosterone levels after numerous weeks of exposure.

In addition, the studies showed dilated and congested blood vessels, hyaline material in interstitial connective tissues, and absence of Leydig cells. These results are discussed by several studies (Barnholt et al., 2006; Okumura et al., 2003). Earlier, Semenza, (2001) which have reported that the increase of vascular endothelial growth factor (VEGF) contents and the representation of their receptors in hypoxic cells are arbitrated by hypoxia inducing factor 1 (HIF-1) (Semenza, 2001). Additionally, Hwang *et al.* (2007) found that VEGF has an effect on the propagation of Leydig cells and on testosterone levels in a dose-dependent manner (Hwang et al., 2007). Environmental hypoxia induces vascular alterations that are related with a rise in the temperature of the testes (Farias et al., 2005; Farías et al., 2008). Recently, Velickovic *et al.* (2012) reported that at the tissue level, a hypoxic condition induces neovascularization that needs a sequence of actions involving differentiation, proliferation and migration of endothelial cells, as well as formation and maturation of vessels. All these are induced through VEGF (Velickovic et al., 2012).

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In the present study, most seminiferous tubules showed disorganization of spermatogenic cells, injured germ cells exiting the basal lamina (with vacuoles in between the spermatogenic cells), absence of Sertoli cells, and reduction in number of sperms with hyaline material in between. Our histopathological results are in agreement with observations of Liao *et al.* (2010) (Liao et al., 2010), who found that hypobaric hypoxia animal models show a reduction in germ cell numbers, extremely vacuolated Sertoli cells, pyknotic germ cells, decrease in Leydig cell number, increase of testicular blood vessels diameter, and alterations in testosterone levels. Degeneration and necrosis of some spermatogenic cells in association with other pathological changes were detected in the testicular tissues of rats whose diet is supplemented with variable concentrations of $NaNO_2$. These findings could be due to a defect in the function of Sertoli cells, as reported previously by Grant and Butler (1989) (Grant and Butler, 1989). Turner and Lysiak (2008) found that experimental testicular ischemia/reperfusion (IR) in rats and mice caused a reduction in germ cells, seminiferous epithelium vacuolization, alleviations in sperm creation, and apoptosis of germ cells (Turner and Lysiak, 2008). The lesions are similar to the results observed in our study.

El-Wakf *et al.* (2009) (El-Wakf et al., 2009) and Ansari *et al.* (2015) (Ansari et al., 2015) reported that $NaNO_2$ induces protein and lipid oxidation, membrane damage, osmotic fragility, and morphological alterations in human erythrocytes. Their data suggested the creation of ROS and resultant production of oxidative stress. The method by which stable reduction in oxygen levels supply or create injury/death of germ cells is likely a rise in intra-testicular ROS. Although ROS possess a physiological function in the spermatogenic procedure, a pathological rise in ROS numbers would negatively impact the endurance and development of germ cells (Ramalho-Santos et al., 2008). The toxic consequences of using $NaNO_2$ are believed to be arbitrated by the formation of oxidative ions. This is reinforced by the explanation that $NaNO_2$ raises lipid peroxidation, and lowers GSH levels and protein oxidation in treated tissues (Vossen and De Smet, 2015). Moreover, $NaNO_2$ toxicity can be alleviated by antioxidant supplementation (Al-Gayyar et al., 2014; Al-Gayyar et al., 2016). Pavlova *et al.*, (2013) found that sperm count reduction was observed in all $NaNO_2$ administration experimental groups (Pavlova et al., 2013); these alterations could be signs of damaged spermatogenesis.

In the present work, biochemical analyses of the testes tissue revealed that $NaNO_2$ toxicity induced a significant elevation of MDA, NO and DNA F%. Lipid peroxidation has been recommended as one of the basic molecular mechanisms for degeneration and necrosis of some of the spermatogenic cells. In addition, other pathological changes were detected in the testicular tissues of rats whose diet was supplemented with variable concentrations of sodium nitrite (Vossen and De Smet, 2015). These findings could be due to defects in the function of Sertoli cells, as reported previously by Grant and Butler (1989) (Grant and Butler, 1989). Additionally, formation of reactive nitrogen species by $NaNO_2$ plays a vital role in its carcinogenic effect on cells or different body tissues- activating

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lipid peroxidation, enzyme inactivation, DNA abrasions and organ injury (El-Wakf et al., 2009). These mechanisms could explain the detection of different pathological changes in various organs examined in the present work.

Lim *et al*. (2014) and Marx *et al*. (2015) also reported that MSCs are believed to be the main promising cell platform for curative applications (Lim et al., 2014; Marx et al., 2015). Nevertheless, the proliferation of MSCs and differentiation capability may vary among species and tissue origin. Numerous studies have showed that O_2 supply might deeply affect stem cell viability and can support certain types of stem differentiation, while preventing other types of differentiation (Simon and Keith, 2008). Spermatogenesis in mammals is a firmly synchronized and incessant process where spermatogonial cells upgrade to the final form of spermatozoa. A physiological hypoxic state keeps self-renewal of spermatogonial cells and spermatogenesis. The vulnerability of the testes to pathological hypo-oxygenic state, particularly chronic hypoxia, is a cause of some types of male infertility (Velickovic and Stefanovic, 2014).

Our histological evaluation of rat testes sections of groups treated with MSCs (G3 and G4 groups) showed an improvement of the testicular structure and a relatively normal order of the different stages of spermatogonia**.** These results are in agreement with Cakici *et al*. (2013) (Cakici et al., 2013), who recorded that MSCs were present in both the external basal partitions and in the internal side of seminiferous tubules. Such observations suggest that MSCs might possess a role in recovering spermatogenesis via two mechanisms: 1) MSC transformation into sperm, or 2) preservation of the spermatogonial cells. Hassan and Alam (2014) showed that a functional rich source for treating infertility is through MSCs (Hassan and Alam, 2014). Yazawa *et al*. (2006) confirmed that MSCs possess the ability to distinguish into steroidogenic cells, like Leydig cells, in vivo and in vitro (Yazawa et al., 2006). Lue *et al*. (2007) revealed that MSCs, injected into testes of infertility busulfan-treated mice, can differentiate into germ cells, Leydig cells and Sertoli cells (Lue et al., 2007).

Conclusion

Treatment with $NaNO_2$ induced hypoxia in the rat testes. $NaNO_2$ led to an increase of NO, MDA contents and DNA F% in testes tissues. In addition, a significant decrease was shown in CAT and TAA activities of testes tissues when compared with control rats. Moreover, the histological hypoxic testes sections showed detachment of basement membrane from spermatogenic layers and widening of seminiferous lumen, faintly stained spermatogenic cell layers in different stages (due to vacuolated cytoplasm), and hydropic degeneration and swing shape of sperm cells. Importantly, treatment of rats with MSCs improves toxicity associated with $NaNO_2$ induced hypoxia in the testes of the rats. Overall, the data indicate that MSC therapy can limit damage from hypoxia induced by $NaNO_2$.

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Abbreviations

CAT Catalse

DNA F% DNA fragmentation percentage

MDA Malondialdhyde

MSCs mesenchymal Stem Cells

NaNO2 Sodium nitrite

NO Nitric oxide

TAA Total antioxidant activity

Author contribution

Prof. Dr Nadia Ismaeil and Dr Amany Osman shared in designing the manuscript and photo and reviewed the histological studies. Prof. Dr. Laila Rashed prepared and suplemented us with the stem cells. Dr. Elham Ali shared in designing the manuscript, interpret the data and reviewed the physiological studies, and Manal Saleh preform the experiment, interpret the data, and write the manuscript.

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Original Research

The incidence and mortality of lip and oral cavity cancer and its relationship to the 2012 Human Development Index of Asia

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Abstract

Introduction: Lip and oral cavity cancer is one of the most prevalent cancers in Asia and considered to be a major public health problem due to the low survival rate. Because of the importance of access to information about this cancer (including incidence, mortality rate and relation to socioeconomic indicators), this study aims at investigating the incidence and mortality of lip and oral cavity cancer and its relationship with the Human Development Index (HDI) of Asia (from 2012). **Method:** This study was an ecological study in Asia for assessment of the correlation between age-specific incidence rate (ASIR) and age-specific mortality rate (ASMR) with the HDI and its components which include: life expectancy at birth, mean years of schooling and gross national income (GNI) per capita. Data on the standardized incidence ratio (SIR) and the standardized mortality ratio (SMR) for every Asian country for the year 2012 were obtained from the global cancer project and data on the HDI and its components were extracted from the World bank site.

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We used a bivariate method for assessment of the correlation between the SIR and SMR with the HDI and its individual components. Statistical significance was assumed if P<0.05. All reported P-values were two-sided. Statistical analyses were performed using SPSS (Version 15.0, SPSS Inc.). **Results:** A total incidence of 162,506 cases and 95,005 deaths were recorded in Asian countries in 2012. Countries with the highest SIR (per 100,000) were the following: Maldives (11), Sri Lanka (10.3), Pakistan (9.8), Bangladesh (9.4), and India (7.2). The highest SMR was observed in the following countries: Pakistan (5.9), Bangladesh (5.6), Afghanistan (5.1), India (4.9), and Maldives (4.1). The correlation between SIR of lip and oral cavity cancer and HDI was -0.378 (p=0.010), with life expectancy at birth at -0.324 (p=0.028), mean years of schooling at -0.283 (p=0.057), and level of income per each person of the population at -0.279 (p=0.060). Moreover, the correlation was -0.664 (p≤0.001**)** between SMR and HDI. **Conclusion:** A significant reverse correlation was seen between the incidence and mortality rate of lip and oral cavity cancer and the HDI in Asia. The incidence and mortality of this type of cancer was high in developing or less developed countries.

Keywords

Lip and oral cavity cancer, Human development index, Incidence, Mortality, Asia

Introduction

Cancers are among the leading causes of disease burden and mortality in the world and are regarded as a significant and growing public health problem around the world (Global Burden of Disease Cancer, 2015; Torre et al., 2015). Among cancers, lip and oral cavity cancer is the result of aggressive tumors originating from external lip and oral cavity and is the eighth most common cancer in men and the fourteenth most common cancer in women worldwide (de Camargo Cancela et al., 2010; Farah et al., 2014). In 2012, 14.1 million new cancer cases and 8.2 million cancer deaths occurred in the world; among them, 300,400 new cases and 145,400 deaths were due to lip and oral cavity cancer, accounting for more than 2% of new cases and 1.7% cases of death in the world, respectively (Torre et al., 2015). Most cancers of the lip and oral cavity have the same preventable risk factors (Warnakulasuriya, 2009). Smoking, alcohol, and chewing tobacco and betel quid (synergistic relationship) are the main risk factors for this type of cancer (Lin et al., 2005; Monteiro et al., 2013; Neville and Day, 2002; Warnakulasuriya, 2009). Poor eating habits, sun overexpsoure, viral infections (particularly human papillomavirus (HPV)), poor oral hygiene, and

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socio-economic factors are also important risk factors for lip and oral cavity cancers (de Camargo Cancela et al., 2010; Farah et al., 2014; Funk et al., 2002; Ribeiro et al., 2015; Warnakulasuriya, 2009).

There is a large geographic variation in the incidence of lip and oral cavity cancer. The highest incidence rates have been reported from Malaysia, South Central Asia, and East and Central Europe, while the lowest rates have been reported from West Africa and East Asia. In recent decades, the incidence rate of lip and oral cavity cancers have decreased in men and women from Asia, North America and Australia, and in men from South and West Europe. However, the rates have increased in men and women from East and North Europe and in women from South and West Europe. The main reasons for this are the rising tobacco epidemic trend as well as the increased prevalence of HPV infection in some countries (Torre et al., 2015; Yako-Suketomo and Matsuda, 2010). Lip and oral cavity cancer is 90% squamous cell carcinoma and is often seen in middle-aged and older people. Its mortality is higher in men and black people, but lower in women (due to less exposure to risk factors such as smoking and alcohol) (de Camargo Cancela et al., 2010; Funk et al., 2002; Neville and Day, 2002; Yako-Suketomo and Matsuda, 2010).

Studies have shown that socioeconomic inequalities which affect behavior and lifestyle have a relation to the incidence and mortality rates of oral cavity cancer. However, some studies have shown conflicting results (Chen et al., 2009; de Camargo Cancela et al., 2010; Patel et al., 2012; Warnakulasuriya, 2009). To review countries' economic and social conditions, various indicators have been defined. One of the most important of the indicators is the Human Development Index (HDI) (Giebel et al., 2010; Hu et al., 2013). This index was first used by the United Nations Development Program and is a combination of three major factors- longevity, knowledge, and standard of living. The HDI is represented as a number between zero and one. Longevity is measured by life expectancy at birth and expressed as Life Expectancy Index. Knowledge is evaluated by a combination of adult literacy rate and the rate of enrollment at primary, secondary and tertiary schools (Education Index). The standard of living is measured by the Gross Domestic Product per capita, with purchasing power parity in US dollars (Gross Domestic Product Index) (Giebel et al., 2010; Hou et al., 2015; Rahi, 2011).

Some studies have shown the relationship between HDI and cancer incidence and mortality (Fidler et al., 2016; Pakzad et al., 2016; Rafiemanesh et al., 2016; Razi et al., 2016). However, to date, no study has been conducted to investigate the relationship between the HDI and the incidence and mortality of lip and oral cavity cancer in Asia. Knowledge of information about the incidence and mortality of lip and oral cavity cancer and its related factors can be useful for planning and developing policies related to health care. This study was aimed to determine the standardized incidence ratio (ASIR) and the standardized mortality ratio (SMR) of lip and oral cavity cancers, and the relationship of ASIR and ASMR with the 2012 HDI of Asian countries.

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Methods

This study was an ecological study in Asia with the goal of assessing the correlation between age-specific incidence and mortality rates of lip and oral cavity cancer with the Human Development Index (HDI) and its components (life expectancy at birth, mean years of schooling, and gross national income per capita. Data about the age-specific incidence and mortality rates for every Asian country for the year 2012 were obtained from the global cancer project available online (http://globocan.iarc.fr/Default.aspx) (Ferlay J S, 2012). The HDI from the Human Development Report of 2013 (Malik, 2013) included information about the HDI and its components for every country in 2012.

A method of age-specific incidence and mortality rates from the global cancer project of the International Agency for Research on Cancer (France) was previously reported (Ferlay et al., 2014; Jemal et al., 2011; Torre et al., 2015).

Human Development Index (HDI)

The Human Development Index (HDI) is derived from a composite measure of indicators along three dimensions: life expectancy at birth, mean years of schooling and level of income per each person of the population (i.e. gross national income per capita) (Malik, 2013).

Statistical analysis

In this study, we used the correlation bivariate method for assessment of correlation between age-specific incidence and mortality rates with HDI and its components (including life expectancy at birth, mean years of schooling and gross national income per capita. Statistical significance was assumed if $P< 0.05$. All reported P-values were two-sided. Statistical analyses were performed using SPSS software (Version 15.0, SPSS Inc.).

Results

Overall, 162,506 cases of lip and oral cavity cancer were recorded in Asian countries in 2012. Of these cases, 106,308 (65.41%) were men and 56,198 cases (34.58%) were women. The sex ratio (male to female) was 1.89. The five countries with the highest number of new cases of lip and oral cavity cancer were:

1) India (77,002 cases),

2) China (21,413 cases),

3) Pakistan (12,761 cases),

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4) Bangladesh (10,550 cases),

5) Japan (8,306 cases).

These 5 countries, collectively, had a sum of 130,033 cases (80.01%).

Of the Asian countries, the 5 countries with the highest standardized incidence rates of lip and oral cavity cancer were:

1) Maldives (standardized rate of 11 per 100,000 people),

2) Sri Lanka (10.3 per 100,000 people),

3) Pakistan (9.8 per 100,000 people),

4) Bangladesh (9.4 per 100,000 people),

5) India (7.2 per 100,000 people).

Conversely, the 5 countries with the lowest standardized rates of lip and oral cavity cancer were:

1) China (1.2 per 100,000 people),

2) Democratic Republic of Korea (1.3 per 100,000 people),

3) Kuwait (1.5 per 100,000 people),

4) Azerbaijan (1.7 per 100,000 people), and

5) Jordan (1.7 per 100,000 people).

The number as well as crude and standardized incidence rates of the cancer, according to sex, of the Asian countries are presented in **Table 1**. The countries are classified from highest to lowest, based on standardized incidence rates. The highest and lowest standardized incidence rates are indicated for both sexes (**Table 1, Fig. 1**).

On the other hand, 195,005 cases of death from of lip and oral cavity cancer have occurred in Asia in 2012. Of the cases, 62,860 (66.16%) were men and 32,145 cases (33.83%) were women. The sex ratio of death from lip and oral cavity cancer in Asian countries was 1.95. Of these, the largest numbers of deaths were seen in:

1) India (52,067 cases),

2) China (11,337 cases),

3) Pakistan (7,766 cases),

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4) Bangladesh (6,571 cases), and

5) Japan (3,994 cases).

These five countries, collectively, had a sum of 80,731 cases (84.97%) of deaths.

Of the Asian countries, the 5 countries with the highest standardized mortality rates of lip and oral cavity cancer were:

1) Pakistan (5.9 per 100,000 people),

2) Bangladesh (5.6 per 100,000 people),

3) Afghanistan (5.1 per 100,000 people),

4) India (4.9 per 100,000 people), and

5) Maldives (4.1 per 100,000 people).

Conversely, the 5 countries with the lowest standardized mortality rates of lip and oral cavity cancer were:

1) Qatar (0.4 per 100,000 people),

2) Kuwait (0.4 per 100,000 people),

3) Bahrain (0.4 per 100,000 people),

4) Oman (0.4 per 100,000 people), and

5) United Arab Emirates (0.5 per 100,000 people).

The number as well as crude and standardized mortality rates of the cancer, according to sex, of the Asian countries are presented in **Table 2**. The countries are classified from highest to lowest, based on standardized mortality rates. The highest and lowest standardized mortality rates are indicated for both sexes (**Table 2, Fig. 1**).

Assessing the relationship between standardized incidence rate and the Human Development Index

Overall, a negative correlation of 0.378 was seen between the standardized incidence rate of lip and oral cavity cancer and the HDI; the correlation was statistically significant (P=0.010). A negative correlation was also seen between components of the HDI and the standardized incidence rate. Moreover, a negative correlation was seen when assessing the relationship of the standardized incidence rate to life expectancy at birth (0.324; P=0.028), to mean age of education (0.283; P=0.057), and to level of income per person of the population (0.279; P=0.060).

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Table 1. Number, crude and standardized incidence rates of lip and oral cavity cancer in Asian countries in 2012 (sorted by age standardized incidence rates of both sexes from highest to lowest)

Lip, oral cavity Estimated incidence, all ages: both sexes				Lip, oral cavity Estimated incidence, all ages: male				Lip, oral cavity Estimated incidence, all ages: female			
POPULATION	Numbers	Crude Rate	ASR (W)	POPULATION	Numbers	Crude Rate	ASR (W)	POPULATION	Numbers	Crude Rate	ASR (W)
Maldives	28	8.6	11.0	Sri Lanka	1845	17.6	15.4	Pakistan	5693	6.4	9.1
Sri Lanka	2667	12.6	10.3	Maldives	20	12.2	15.4	Brunei	9	4.4	9.0
Pakistan	12761	7.1	9.8	Bangladesh	7120	9.2	13.0	Maldives	8	5.0	6.4
Bangladesh	10550	6.9	9.4	Kazakhstan	788	10.0	11.6	Bangladesh	3430	4.6	5.9
India	77003	6.1	7.2	Pakistan	7068	7.7	10.5	Sri Lanka	822	7.6	5.7
Kazakhstan	1083	6.6	6.3	India	53842	8.3	10.1	Afghanistan	435	2.7	5.4
Afghanistan	1047	3.1	6.3	Turkmenistan	172	6.8	9.3	Timor-Leste	19	3.3	5.3
Myanmar	2775	5.7	6.2	Myanmar	1810	7.5	8.6	Cambodia	304	4.1	5.2
Brunei	18	4.4	6.0	Nepal	701	4.6	7.2	India	2316	3.8	4.3
Cambodia	584	4.0	6.0	Afghanistan	612	3.5	7.1	Lao PDR	91	2.9	4.2
Timor-Leste	40	3.4	5.6	Cambodia	280	3.9	7.1	State of Palestine	46	2.2	4.1
Turkmenistan	224	4.3	5.6	Armenia	116	8.0	6.7	Myanmar	965	3.9	4.1
Nepal	942	3.0	4.4	Timor-Leste	21	3.5	5.9	Philippines	1105	2.3	3.2
Kyrgyzstan	163	3.0	4.0	Kyrgyzstan	108	4.0	5.8	Thailand	1551	4.4	3.0
Thailand	3709	5.3	4.0	Thailand	2158	6.3	5.1	Kazakhstan	295	3.5	2.8
State of Palestine	90	2.1	3.8	Georgia	159	7.8	4.9	Malaysia	363	2.5	2.8
Philippines	2363	2.4	3.6	Brunei	9	4.3	4.5	Kyrgyzstan	55	2.0	2.5
Armenia	165	5.3	3.6	Bhutan	14	3.5	4.3	Turkmenistan	52	2.0	2.4
Lao PDR	140	2.2	3.4	Uzbekistan	425	3.0	4.2	Yemen	157	1.2	2.3
Bhutan	19	2.5	3.2	Philippines	1258	2.6	4.1	Nepal	241	1.5	2.1
Malaysia	776	2.6	3.0	Japan	4881	7.9	3.9	Saudi Arabia	164	1.3	2.1
Uzbekistan	661	2.4	3.0	Tajikistan	75	2.2	3.7	Israel	116	3.0	2.0

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Japan	8306	6.6	2.9	Singapore	125	4.7	3.4	Bhutan	5	1.4	2.0
Tajikistan	123	1.7	2.7	State of Palestine	44	2.0	3.4	Japan	3425	5.3	2.0
Georgia	205	4.8	2.6	Malaysia	413	2.8	3.3	Uzbekistan	236	1.7	1.9
Singapore	190	3.6	2.5	Viet Nam	1392	3.1	3.3	Indonesia	2327	1.9	1.9
Viet Nam	2147	2.4	2.4	Bahrain	14	1.6	3.2	Qatar	3	0.6	1.9
Indonesia	5329	2.2	2.3	Iraq	227	1.3	3.0	Tajikistan	48	1.3	1.8
Iraq	411	1.2	2.3	Korea, Republic of	965	4.0	2.9	Iran, Islamic Republic of	617	1.7	1.8
Bahrain	18	1.3	2.2	Indonesia	3002	2.5	2.8	Mongolia	18	1.2	1.8
Korea, Republic of	1575	3.2	2.2	Turkey	939	2.5	2.7	Iraq	184	1.1	1.7
Israel	231	3.0	2.2	Syrian Arab Republic	185	1.7	2.7	Singapore	65	2.5	1.7
Yemen	283	1.1	2.2	Lebanon	54	2.6	2.6	Oman	12	1.0	1.7
United Arab Emirates	80	1.0	2.1	Lao PDR	49	1.5	2.5	Korea, Republic of	610	2.5	1.6
Qatar	23	1.2	2.1	Israel	115	3.0	2.4	Viet Nam	755	1.7	1.6
Turkey	1502	2.0	2.1	Azerbaijan	97	2.1	2.4	Lebanon	37	1.7	1.5
Iran, Islamic Republic of	1380	1.8	2.0	United Arab Emirates	59	1.1	2.4	United Arab Emirates	21	0.8	1.5
Syrian Arab Republic	301	1.4	2.0	Iran, Islamic Republic of	763	2.0	2.2	Turkey	563	1.5	1.5
Saudi Arabia	358	1.2	2.0	Jordan	47	1.4	2.2	Syrian Arab Republic	116	1.1	1.5
Lebanon	91	2.1	2.0	Mongolia	19	1.4	2.2	Armenia	49	2.9	1.4
Mongolia	37	1.3	2.0	Korea, Democratic Republic of	283	2.3	2.1	Kuwait	9	0.8	1.3
Oman	34	1.2	1.8	Saudi Arabia	194	1.2	2.0	Jordan	27	0.9	1.2
Jordan	74	1.1	1.7	Qatar	20	1.4	2.0	Bahrain	4	0.8	1.2
Azerbaijan	160	1.7	1.7	Yemen	126	1.0	2.0	Azerbaijan	63	1.3	1.1
Kuwait	25	0.9	1.5	Oman	22	1.3	1.9	Georgia	46	2.0	0.9
Korea, Democratic Republic of	402	1.6	1.3	China	13656	1.9	1.6	China	7757	1.2	0.9
China	21413	1.6	1.2	Kuwait	16	0.9	1.5	Korea, Democratic Republic of	119	1.0	0.7

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In men, a negative correlation of 0.323 was seen between the standardized incidence rate of lip and oral cavity cancer and the HDI; the correlation was statistically significant (P=0.029). A negative correlation was also seen between components of the HDI and the standardized rate. Moreover, a negative correlation was seen when assessing the relationship of the standardized incidence rate to life expectancy at birth (0.279; P=0.061), to mean age of education (0.167; P=0.267), and to level of income per person of the population (0.323; P=0.029).

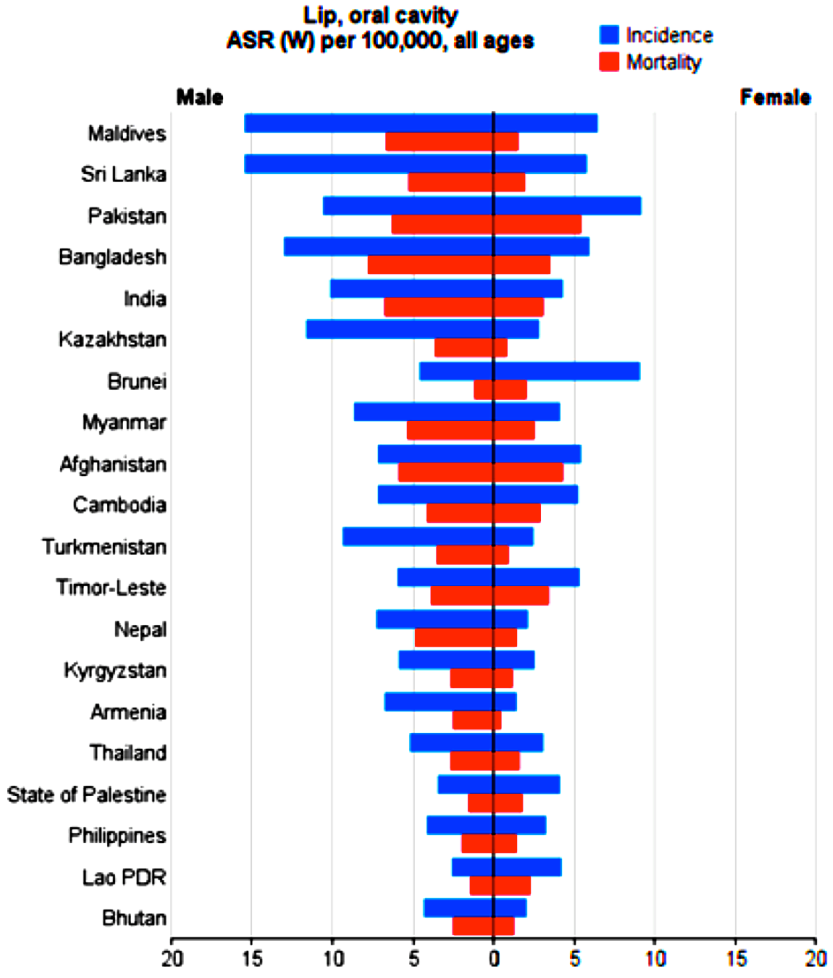

Figure 1. Standardized incidence and mortality rates of lip and oral cavity cancer in Asia in 2012 (extracted from GLOBOCAN 2012).

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In women, a negative correlation of 0.337 was seen between the standardized incidence rate of lip and oral cavity cancer and the HDI; the correlation was statistically significant (P=0.022). A negative correlation was also seen between components of the HDI and the standardized rate. Moreover, a negative correlation was seen when assessing the relationship of the standardized incidence rate to life expectancy at birth (0.310; P=0.036), to mean age of education (0.348, P=0.018), and to level of income per person of the population (0.132; P=0.382).

Assessing the relationship between standardized mortality rate and the Human Development Index

Overall, a negative correlation of 0.664 was seen between the standardized mortality rate of lip and oral cavity cancer and the HDI; the correlation was statistically significant (P≤0.001). Also, a significant negative correlation was seen between components of the HDI and the standardized rate. In fact, a negative correlation was seen when assessing the relationship of the standardized mortality rate to life expectancy at birth (0.592; P≤0.001), to mean age of education (0.528; P≤0.001), and to level of income per person of the population (0.421; P=0.004).

In men, a negative correlation of 0.603 was seen between the standardized mortality rate of lip and oral cavity cancer and the HDI; the correlation was statistically significant (P≤0.001). Also, a significant negative correlation was seen between components of the HDI and the standardized rate. In fact, a negative correlation was seen when assessing the relationship of the standardized mortality rate to life expectancy at birth (0.518; P≤0.001), to mean age of education (0.448; P=0.002), and to level of income per person of the population (0.429; P=0.003).

In women, a negative correlation of 0.666 was seen between the standardized mortality rate of lip and oral cavity cancer and the HDI; the correlation was also statistically significant (P≤0.001). Moreover, a significant negative correlation was seen between components of the HDI and the standardized rate. In fact, a negative correlation was seen when assessing the relationship of the standardized mortality rate to life expectancy at birth (0.639; P≤0.001), to mean age of education (0.559; P≤0.001), and to level of income per person of the population (0.365; P≤0.001).

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Table 2. Number, crude and standardized mortality rates of lip and oral cavity cancer in Asian countries in 2012 (sorted by age standardized rates of both sexes from highest to lowest)

Lip, oral cavity - Estimated mortality, all ages: both sexes				Lip, oral cavity - Estimated mortality, all ages: female				Lip, oral cavity - Estimated mortality, all ages: male			
POPULATION	Numbers	Crude Rate	ASR (W)	POPULATION	Numbers	Crude Rate	ASR (W)	POPULATION	Numbers	Crude Rate	ASR (W)
Pakistan	7266	4.0	5.9	Bangladesh	4094	5.3	7.7	Pakistan	3220	3.6	5.4
Bangladesh	6071	4.0	5.6	India	36436	5.6	6.7	Afghanistan	318	2.0	4.3
Afghanistan	771	2.3	5.1	Maldives	8	4.9	6.6	Bangladesh	1977	2.6	3.5
India	52067	4.1	4.9	Pakistan	4046	4.4	6.3	Timor-Leste	11	1.9	3.4
Maldives	10	3.1	4.1	Afghanistan	453	2.6	5.8	India	15631	2.6	3.0
Myanmar	1668	3.4	3.8	Myanmar	1090	4.5	5.3	Cambodia	165	2.2	2.9
Timor-Leste	23	1.9	3.6	Sri Lanka	634	6.1	5.2	Myanmar	578	2.3	2.5
Sri Lanka	916	4.3	3.5	Nepal	451	2.9	4.8	Lao PDR	48	1.5	2.3
Cambodia	316	2.2	3.4	Cambodia	151	2.1	4.1	Brunei	2	1.0	2.0
Nepal	606	2.0	2.9	Timor-Leste	12	2.0	3.8	Sri Lanka	282	2.6	1.9
Thailand	1913	2.7	2.1	Kazakhstan	238	3.0	3.6	State of Palestine	17	0.8	1.7
Turkmenistan	79	1.5	2.1	Turkmenistan	60	2.4	3.5	Thailand	799	2.2	1.6
Kazakhstan	325	2.0	1.9	Kyrgyzstan	44	1.6	2.6	Yemen	99	0.8	1.6
Lao PDR	74	1.2	1.9	Thailand	1114	3.2	2.6	Maldives	2	1.2	1.5
Bhutan	10	1.3	1.8	Bhutan	7	1.8	2.4	Nepal	155	1.0	1.4
Kyrgyzstan	69	1.3	1.8	Armenia	41	2.8	2.4	Philippines	451	0.9	1.4
State of Palestine	35	0.8	1.6	Philippines	527	1.1	1.9	Bhutan	3	0.9	1.2
Philippines	978	1.0	1.6	Uzbekistan	173	1.2	1.8	Kyrgyzstan	25	0.9	1.1
Yemen	180	0.7	1.5	Tajikistan	34	1.0	1.7	Turkmenistan	19	0.7	0.9
Brunei	4	1.0	1.4	Mongolia	14	1.0	1.7	Mongolia	8	0.6	0.9
Uzbekistan	270	1.0	1.3	Iraq	107	0.6	1.5	Iraq	87	0.5	0.8
Tajikistan	55	0.8	1.2	Viet Nam	632	1.4	1.5	Uzbekistan	97	0.7	0.8
Armenia	58	1.9	1.2	State of Palestine	18	0.8	1.5	Tajikistan	21	0.6	0.8

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Mongolia	22	0.8	1.2	Georgia	49	2.4	1.4	Indonesia	987	0.8	0.8
Iraq	194	0.6	1.1	Japan	2188	3.6	1.4	Kazakhstan	87	1.0	0.8
Viet Nam	971	1.1	1.1	Lao PDR	26	0.8	1.4	Malaysia	97	0.7	0.8
Japan	3994	3.2	1.1	Yemen	81	0.6	1.3	Viet Nam	339	0.7	0.7
Indonesia	2250	0.9	1.0	Malaysia	156	1.0	1.2	Japan	1806	2.8	0.7
Malaysia	253	0.9	1.0	Indonesia	1263	1.0	1.2	Kuwait	4	0.3	0.7
Syrian Arab Republic	119	0.6	0.8	Brunei	2	1.0	1.1	Iran, Islamic Republic of	200	0.5	0.6
Georgia	63	1.5	0.8	Syrian Arab Republic	73	0.7	1.1	Syrian Arab Republic	46	0.4	0.6
Singapore	58	1.1	0.7	Singapore	41	1.5	1.1	Saudi Arabia	44	0.3	0.6
Turkey	503	0.7	0.7	Korea, Democratic Republic of	138	1.1	1.0	Turkey	187	0.5	0.5
Iran, Islamic Republic of	449	0.6	0.7	Korea, Republic of	339	1.4	1.0	Israel	35	0.9	0.5
Korea, Republic of	517	1.1	0.6	Turkey	316	0.9	0.9	United Arab Emirates	5	0.2	0.5
Korea, Democratic Republic of	200	0.8	0.6	China	7370	1.0	0.8	Lebanon	11	0.5	0.4
China	11333	0.8	0.6	Azerbaijan	33	0.7	0.8	Armenia	17	1.0	0.4
Saudi Arabia	97	0.3	0.6	Lebanon	16	0.8	0.8	Jordan	8	0.3	0.4
Israel	70	0.9	0.6	Iran, Islamic Republic of	249	0.6	0.7	China	3963	0.6	0.4
Lebanon	27	0.6	0.6	Jordan	15	0.5	0.7	Singapore	17	0.7	0.4
Jordan	23	0.4	0.6	Israel	35	0.9	0.7	Korea, Republic	178	0.7	0.4
Azerbaijan	54	0.6	0.6	Saudi Arabia	53	0.3	0.6	Azerbaijan	21	0.4	0.3
United Arab Emirates	19	0.2	0.5	Oman	7	0.4	0.6	Korea, Democratic Republic of	62	0.5	0.3
Oman	8	0.3	0.4	Qatar	5	0.3	0.5	Georgia	14	0.6	0.3
Bahrain	4	0.3	0.4	Bahrain	3	0.4	0.5	Bahrain	1	0.2	0.2
Kuwait	8	0.3	0.4	United Arab Emirates	14	0.2	0.5	Oman	1	0.1	0.1
Qatar	5	0.3	0.4	Kuwait	4	0.2	0.3	Qatar	0	0.0	0.0

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Discussion

Although lip and oral cavity cancer accounts for less than 3% of all cancer cases worldwide, its low survival rate and adverse consequences on quality of life have garnered it to be considered as a significant public health problem; in fact, two thirds of its burden occurs in developing countries (Costa et al., 2016; Farah et al., 2014; Global Burden of Disease Cancer, 2015; Ribeiro et al., 2015; Torre et al., 2015; Warnakulasuriya, 2009). Studies show that 162,506 new cases of lip and oral cavity cancer have been recorded in Asia in 2012, accounting for 56.1% of all new cancer cases worldwide in 2012. There was a significant inverse relationship between the lip and oral cavity cancer and the HDI in Asia. The highest standardized incidence rates for this type of cancer, among the Asian countries, were seen in Maldives, Sri Lanka, Pakistan, Bangladesh and India, respectively. These countries were among the countries with medium HDI.

Since people who live in developing countries are exposed to a wider range of risk factors for cancer of the lip and oral cavity, the highest incidence rates are reported from these countries (Byakodi et al., 2012; de Camargo Cancela et al., 2010; Gupta et al., 2016; Rastogi et al., 2004). The most important risk factors of this cancer are tobacco use, alcohol, chewing tobacco, betel quid, poor eating habits, sun exposure, viral infections (especially HPV), and poor oral hygiene (de Camargo Cancela et al., 2010; Farah et al., 2014; Funk et al., 2002; Ribeiro et al., 2015; Warnakulasuriya, 2009). In India and Pakistan, about 100 million people use various types of smokeless tobacco and betel-quid chewing (Jayalekshmi et al., 2009). In addition to these countries, these tobacco and chewing habits are also common in Bangladesh, Afghanistan, Maldives, Sri Lanka and Nepal, which has led to an increased risk of lip and oral cavity cancers in these areas (Ariyawardana and Warnakulasuriya, 2011; Funk et al., 2002; Khan et al., 2016; Neville and Day, 2002; Sreeramareddy et al., 2014).

In the present study, an inverse relationship was seen between the incidence of lip and oral cavity cancer and the HDI components. The correlation was significant for life expectancy but insignificant for education and income. Studies have shown that the incidence of lip and oral cavity cancer is higher in people with lower education and income (de Camargo Cancela et al., 2010; Farah et al., 2014; Jayalekshmi et al., 2009; Johnson et al., 2010; Ribeiro et al., 2015; Swaminathan et al., 2009). People with less education are at greater risk of lip and oral cavity cancer due to less awareness of cancer risk factors, poor sanitary habits, greater consumption of alcohol and tobacco, and use of chewing tobacco (Gupta et al., 2016; Hashibe et al., 2003; Videnovic et al., 2016; Warnakulasuriya, 2009). Also, people with less income are more likely to have this type of cancer due to limited access to dental care, poor oral hygiene, consumption of fewer fruits and vegetables, greater HPV risk, and less protection against the sun (Arnold et al., 2016; Farah et al., 2014; Guha et al., 2007; Johnson et al., 2010; Monteiro et al., 2013; Morris et al., 2000; Pavia et al., 2006). Chen and colleagues also found an inverse relationship between income

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per capita and the incidence of lip and oral cavity cancer (Chen et al., 2009). In a systematic review and meta-analysis, done by Conway et al. on 41 case control studies from all around the world, economic and social conditions were found to be risk factors for oral cancer. These socioeconomic conditions included: low educational attainment ((odds ratio (OR): 1.85, 95% confidence interval (CI): 1.60–2.15)), low occupational social class (OR: 1.84, 95% CI: 1.47–2.31), and low income (OR: 2.41, 95% CI: 1.59–3.65) (Conway et al., 2008).

Based on the data from Asia, 95,005 deaths occurred due to lip and oral cavity cancer in 2012, which was equivalent to 66.9% of all cancer deaths in the world that year. A significant inverse relation was seen between lip and oral cavity cancer mortality and the HDI. Asian countries with the highest standardized mortality rate from lip and oral cavity cancer were Pakistan, Bangladesh, Afghanistan, India and Maldives, respectively. Afghanistan had low HDI while the rest had medium HDI. The findings showed a significant inverse relation between mortality from lip and oral cavity cancer and the HDI components (including life expectancy, education and income). Studies have shown that less education, lack of awareness about the symptoms of lip and oral cavity cancer, and delayed diagnosis are all factor which contribute to higher mortality rates (Albano et al., 2007; Kilander et al., 2001; Warnakulasuriya, 2009).

Despite advances in medical sciences, over the past several decades the overall five-year survival rate for lip and oral cavity cancer has not improved significantly, remaining at about 50-55% (Neville and Day, 2002; Warnakulasuriya, 2009). In studies that were conducted in Asia, the overall five-year survival rate was 18% in Malaysia (Razak et al., 2010), 30.5 % in India (Yeole et al., 2003), 52.8% in Korea (Choi et al., 2014), and 61% in Taiwan (Liu et al., 2010). Due to limited access to diagnostic and treatment services in low-income communities and to the high cost of services, people present with advanced stage lip and oral cavity cancer at the time of diagnosis. All the aforementioned are among the important reasons for the low 5-year survival of patients as well as the higher mortality rates in developing countries (Funk et al., 2002; Global Burden of Disease Cancer, 2015; Patel et al., 2012; Sargeran et al., 2008). McDonald et al. reported in their study that there was lower survival of head and neck cancers, including oral cavity cancer, in people of low socioeconomic statuses (McDonald et al., 2014).

Mortality and high burden of lip and oral cavity cancer, particularly in developing countries, continues to warrant public education. Awareness about the risk factors and symptoms of lip and oral cavity cancer, screening of high-risk groups, and planning for preventative measures can help the population most at risk for this kind of cancer and will be essential for effective prevention (de Camargo Cancela et al., 2010; Warnakulasuriya, 2009).

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Conclusion

In general, a significant inverse correlation was observed between the incidence of lip and oral cavity cancer and the HDI in Asia. Moreover, the incidence of this cancer was higher in developing countries. This correlation was also observed between cancer incidence and the HDI components; it was significant for life expectancy but insignificant for education and income. A significant inverse correlation was observed between deaths from lip and oral cavity cancer and the HDI and its components, and the mortality rate from this cancer was higher in developing countries.

Abbreviations

HDI: Human Development Index:

ASIR: Age-specific incidence rate

ASMR: Age-specific mortality rate

HPV: Human papillomavirus

Acknowledgement

Data on CANCER were obtained from the global cancer project and data on the HDI and its components were extracted from the World Bank site. Hereby we appreciate of the cooperation of all employees involved in data collection in the GLOBOCAN project and World Bank

Author contribution

All authors contributed to the design of the research. AMH, EI and HSG collected the data. AMH, EI and HS conducted analysis and interpretation of data. All authors drafted the first version. HS, AT and AMH edited the first draft. All authors reviewed and commented on final draft.

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Short Communication

Rationale for stem cell therapy for type 2 diabetes mellitus

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Abstract

Stem cells can be differentiated into many types of mature cells. Among degenerative diseases, type 1 diabetes mellitus (T1DM) is considered to be a good target disease for stem cell therapeutic application. Indeed, several studies have suggested that stem cells can be differentiated, both *in vitro* and *in vivo*, into beta cells which regenerate the pancreas. However, recent studies have shown that stem cell therapy can also provide benefits for type 2 diabetes mellitus (T2DM), which is not related to beta cell degeneration in the pancreas. This commentary will discuss the opportunity to use mesenchymal stem cells (MSCs) to treat T2DM, citing various stem cell therapies from recent published studies. Indeed, a current report *"Expanded autologous adipose derived stem cell transplantation for type 2 diabetes mellitus, Biomedical Research and Therapy, 3(12): 1034-1044"* evaluated and confirmed the positive effects of stem cell transplantation for blood glucose regulation in T2DM.

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Keywords

Stem cells; Mesenchymal stem cells; Type 2 diabetes mellitus, T2DM

T1DM versus T2DM

Diabetes mellitus (DM) is a metabolic disease that causes the accumulation of high glucose levels in the blood. Patients with DM usually exhibit particular symptoms, including frequent urination, increased thirst and increased hunger (in the early phase of the disease), and complications such as diabetic ketoacidosis, non-ketotic hyperosmolar coma, heart disease, stroke, chronic

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kidney failure, foot ulcers and damage to the eyes (in the late phase). The high blood glucose level in DM patients relates to reduction of insulin; beta cells in the pancreas cannot produce enough insulin for usage or cells in the body cannot properly respond to insulin.

Based on differences in mechanisms, DM can be classified into 2 main types: Type 1 DM (T1DM) and Type 2 DM (T2DM). T1DM results when beta cells cannot produce enough insulin for cells of the body. This condition is considered akin to an autoimmune disease since beta cells are destroyed by the immune system. T1DM is common in children and, therefore, is also known as juvenile diabetes. T2DM is different from T1DM in that the beta cells of T2DM patients are able to produce enough insulin; however, the cells of the body cannot use this form of insulin and are said to be "insulin resistant". Since T2DM is not related to insulin; it is regarded as a non-insulin dependent diabetes mellitus.

T1DM, as a degenerative disease, has rapidly attracted stem cell scientists to develop new stem cell based approaches in attempts to treat it. Indeed, T1DM has been viewed by two different perspectives, including as a degenerative disease which lacks beta cells and as an autoimmune disease in which the immune system attacks the beta cells. For this first strategy, both stem cells and insulin-producing cells differentiated from stem cells were used to treat T1DM in both preclinical trials and clinical trials (Xv et al., 2016).

T2DM treatment by stem cell transplantation

Recently, the Pham group reported some cases of T2DM treatment using autologous adipose derived stem cells (ADSCs) (Le et al., 2016). In their study, adipose tissue was collected from patients. ADSCs were extracted and expanded from adipose tissue. The cells were confirmed to be normal stem cells with normal karyotype, and negative for endotoxin and mycoplasma. The T2DM patients received a transplant of ADSCs at a dose of 1-2.10^6 cells/kg. The results showed that after 3 months, blood glucose levels of patients were significantly reduced and there were no adverse effects. From literature searches, only 3 other clinical studies were found which used stem cells to treat T2DM.

There have been two clinical trials using autologous bone marrow mononuclear stem cells (BM-MNSCs). The first clinical trial was performed in 6 patients who were infused with BM-MNSCs in the celiac and superior mesenteric arteries, without any myeloablative or immunosuppressive pretreatment (Wehbe et al., 2016). The results showed that 5/6 patients (83%) showed an increase of fasting glucose levels and a decrease of glycosylated hemoglobin (HbA1C), significantly reducing their medication requirements. Of note, 3/6 patients (50%) showed improvement in diabetic complications and did not show any significant adverse effects (Wehbe et al., 2016).

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The second clinical study also used BM-MNSCs to treat T2DM patients. Here, patients were confirmed as T2DM, for greater than 5 years, with oral antidiabetic drugs along with insulin levels (≥0.4 IU/Kg/day) and HbA1c ≤7.5% (≤58.0 mmol/mol) (Bhansali et al., 2017). The results also showed that after 1 year of follow-up, the primary endpoint showed a reduction in insulin requirement by ≥50% from baseline, while maintaining HbA1c <7.0% (<53.0 mmol/mol). Specifically, 6 of 10 (60%) treated patients achieved the primary endpoint and a significant reduction in insulin requirement at month 12 (Bhansali et al., 2017). In this study, Bhansali et al. (2017) also used bone marrow derived mesenchymal stem cells (BM-MSCs) to treat these T2DM patients. The authors in that publication showed that treatment efficacy of transplanted BM-MSCs is equivalent to transplanted BM-MNCs (Bhansali et al., 2017).

In another study, Liu et al. (2016) reported using MSCs derived from amniotic membrane to treat T2DM patients, with 3 years of follow-up (Liu et al., 2016). This study was carried out in 4 patients. These patients were transplanted with allogeneic neonatal amniotic membrane derived MSCs at $2x10^7$ cells. The results showed that there was an improvement of glycemic control. Indeed, 100% of the patients did not require insulin but did need metformin (250-500 mg/day) to control blood glucose levels. However, by this therapy, there was no effect on C-peptide. Therefore, the authors suggested that there was an increase of insulin sensitivity (Liu et al., 2016).

How can stem cells treat T2DM?

The specific mechanism of MSCs in T2DM treatment remains unclear. However, positive benefits from clinical treatment with MSC have suggested and led to investigations of mechanisms in T2DM patients. Some clinical trials have examined autologous versus allogeneic MSCs, and MSCs versus MNCs. Transplantation with each of them led to improvements in T2DM patients. Indeed, in allogenic transplantation of stem cells, grafted cells could not survive in the host for a long time. Therefore, some effects of stem cell transplantation cannot be from the homing or from differentiation of stem cells into insulin producing cells. Moreover, in the T2DM patients, the human body does not lack insulin. The study of Liu et al. (2016) showed that there was no change in C-peptide concentrations, although there were improvements in blood glucose levels of T2DM patients. Thus, it is likely that stem cell transplantation cannot improve insulin production in T2DM patients.

However, stem cell transplantation may improve the insulin sensitivity of cells in certain tissues. To date, the reason for insulin resistance in T2DM patients has not been elucidated. Some recent studies have suggested that immune reactions, similar to those observed in autoimmune diseases, can cause insulin resistance to be observed in T2DM patients. Indeed, Antuna-Puente et al. (2008) and Sell et al. (2012) showed that chronic inflammation of adipose tissues

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significantly contributed to insulin resistance (Antuna-Puente et al., 2008; Sell et al., 2012). In some studies, it was also shown that insulin resistance in T2DM originated from inflammation. The inflammation could be related to abnormalities of lymphocytes, eosinophils, mast cells and dendritic cells (DeFuria et al., 2013; Liu et al., 2009; Musilli et al., 2011; Talukdar et al., 2012; Wu et al., 2011). If T2DM is an immune-related disease, MSC transplantation seems to be a suitable platform for T2DM therapy. Indeed, MSCs exert their immune modulation thorough suppressing T cells, B cells, natural killer (NK) cells and dendritic cells (DCs) (Le et al., 2016; Pham et al., 2016; Sangiorgi and Panepucci, 2016).

Conclusion

Although the study of MSC transplantation for T2DM is still young, there is great potential for its use, particularly for treating T2DM. Current breakthroughs in the understanding of mechanisms and biological characteristics of MSCs have suggested that MSC transplantation can be effective for T2DM. MSCs can persist *in vivo* without homing or and can differentiate into beta cells to produce insulin in the patients; however, grafted/transplanted cells can improve insulin resistance. Although the mechanism by which MSCs can improve insulin resistance remains unclear, its role in immune modulation to reduce local inflammation and insulin resistance are evident. Based on the benefits of MSCs, transplantation of MSCs may be a highly promising therapy for T2DM treatment.

Abbreviations

BM-MNCs: Bone marrow mononuclear cells

DCs: Dendritic cells

MSC: Mesenchymal stem cell

NK: Natural Killer cell

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

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