Isotope effect of ^{13}C-enriched testosterone on human cells

MENGQI ZHANG[1], XINCHEN WU[2], WENNING YANG[3] and TENGFEI ZHANG[2]

[1]Department of Pediatrics, Harlem Hospital Center, Affiliated with Columbia University, New York, NY 10037, USA; [2]Sanbo Brain Hospital, Capital Medical University, Beijing 100093; [3]School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 102488, P.R. China

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Abstract. The present study investigated the carbon isotope effect of carbon-13 (^{13}C)-enriched testosterone on human cells; studies on the carbon isotope effects of a bioactive compound are limited. For this purpose, human osteoblasts, aortic endothelial cells and umbilical vein endothelial cells were treated with testosterone and ^{13}C-enriched testosterone. The cell growth rates and bioactivities of osteoblasts were measured. At physiological concentrations, testosterone promoted cell proliferation, whereas ^{13}C-enriched testosterone exerted a neutral and concentration-independent effect on the cells. On the whole, the findings of the present study demonstrated that ^{13}C-enriched testosterone exerted a significant isotope effect on human cells; this needs to be taken into account in stable isotope-based biology research.

Introduction

Stable isotope labeling plays a staple role in biological research. As such studies are commonly linked to the kinetic features of biochemical reactions, the kinetic isotope effect (KIE) on the growth and metabolism of an organism should be considered (1,2). An isotope is a species of an element with a different mass due to the different number of neutrons. The isotope effect refers to the phenomenon that the physical and chemical properties of isotopic atoms differ due to the dissimilarities in their nuclear properties (3,4).

For decades, various efforts have been made to explore the biology isotope effects of biogenic elements. Despite the increasing knowledge of the biological isotope effect, a comprehensive understanding of the effects in the biological context has not yet been achieved (2,5-9). Among the main biogenic elements (C, H, O and N), deuterium has been investigated in a number of studies and its effects on organisms have been demonstrated (5-9). However, the carbon isotope effects of bioactive compounds have yet not been investigated, at least to the best of our knowledge, although such studies are important to the biology development in theory and application. Additionally, fewer isotope effect studies on human cells have been conducted compared with other organism (10).

In the present study, testosterone, a typical androgen, was isotopically modified. It was demonstrated that the level of isotope enrichment potentially influenced its isotope effect, and it was classified as high (>50%) and low (<10%) level (11,12). Herein, carbon-13 (^{13}C) was enriched in testosterone with a low enrichment level (^{13}C/^{12}C, 6.7%) in order to investigate the carbon isotope effect on human osteoblasts, aortic endothelial cells and umbilical vein endothelial cells. Androgen is involved in the regulation of a number of physiological processes, including bone development (13,14) and the modulation of vascular behavior (15-17). Osteoblasts and vascular endothelial cells express androgen receptor and are targets for the action of androgen (18-20). Several studies have demonstrated that testosterone enhances the proliferation of human osteoblasts (13), human primary aortic endothelial cells (15,20) and human umbilical vein endothelial cells (21). The present study examined the isotope effect of ^{13}C-enriched testosterone at various concentrations on the growth of the aforementioned cell types. The alkaline phosphatase (ALP) level and osteocalcin (OC) secretion of osteoblasts were examined. To the best of our knowledge, the present study is the first investigate the carbon isotope effect of a bioactive compound on normal human cells.

The aim of the present study was to highlight the importance of isotope effects in stable isotope-based research by investigating the carbon isotope effect of ^{13}C-enriched testosterone on the growth of human cells. The concentration effects at physiological (10^{-10} and 10^{-8} mol/l) (22) and supraphysiological (10^{-6} and 10^{-5} mol/l) levels were investigated using an *in vitro* model.

Materials and methods

Materials and reagents. Human osteoblasts (cat no. 4610), human primary aortic endothelial cells (cat no. H-6052) and human umbilical vein endothelial cells (cat no. C0035C) were obtained from ScienCell Research Laboratories, Inc., Cell Biologics, Inc. and Thermo Fisher Scientific, Inc., respectively. Ascorbic acid, glycerol-2-phosphate and dexamethasone were purchased from

Correspondence to: Dr Xinchen Wu, Sanbo Brain Hospital, Capital Medical University, 50 Xiangshan Yikesong Road, Haidian, Beijing 100093, P.R. China
E-mail: xinchenwu1990@163.com

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Sigma-Aldrich; Merck KGaA. Dulbecco's modified Eagle's medium (DMEM) with low glucose, penicillin-streptomycin, trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Thermo Fisher Scientific, Inc. Testosterone and testosterone-3,4-$^{13}C_2$ were purchased from Alta Scientific. Co., Ltd.

Preparation of ^{13}C-enriched testosterone. Testosterone and testosterone-3,4-$^{13}C_2$ were both dissolved in ethanol and then mixed together at a ratio of 1:1 (mole), by which ^{13}C-enriched testosterone was obtained. The carbon isotopic composition of ^{13}C-enriched testosterone was calculated to be $^{13}C/^{12}C$=6.7%. The chemical structures of testosterone and testosterone-3,4-$^{13}C_2$ are presented in Fig. 1.

Cell culture and compound intervention. Human osteoblasts, human primary aortic endothelial cells and human umbilical vein endothelial cells were thawed and cultured in low-glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin with 5% CO_2 at 37˚C. The cells were dissociated with trypsin-EDTA and seeded in 96-well tissue culture plates at the density of $1x10^4$ cells/well. The culture media were then changed to media with low-glucose DMEM, 10% FBS, 1% penicillin-streptomycin, 50 mg/ml ascorbic acid, 0.01 mol/l glycerol-2-phosphate and 100 nmol/l dexamethasone. The cells were cultured in the culture medium containing either testosterone or ^{13}C-enriched testosterone at concentrations of 0, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l. An untreated (no drugs; 0 mol/l) was used as a blank control. The morphology of the cells treated with testosterone and ^{13}C-enriched testosterone at the concentration of 10^{-5} mol/l was also observed. The observation was conducted using an inverted microscope (XDS-500C, Shanghai Caikon Optical Instrument Co., Ltd.).

Measurement of cell proliferative activity. The proliferative activities of human osteoblasts, human primary aortic endothelial cells and human umbilical vein endothelial cells were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (MTS cell proliferation colorimetric assay kit, AmyJet Scientific, Inc.) when the cells were cultured for 48 h at 37˚C. Following the manufacturer's instructions, MTS and phenazine methosulfate (PMS) solution were mixed (MTS:PMS=20:1). The culture medium and the mixed solution of MTS and PMS were added to the test well of a 96-well plate and were incubated at 37˚C for 2 h. The light absorbance of the formazan product was measured at a 490-nm wavelength using a spectrophotometer (BioTek Instruments, Inc.). The measurement for each concentration was repeated 6 times, and the average optical density (OD) value was recorded.

Measurement of the ALP level of osteoblasts. In order to further investigate the isotope effect of ^{13}C-enriched testosterone on osteoblasts, the ALP level in osteoblasts was determined. An elevation in ALP levels serves as a marker of osteogenic differentiation (23,24). The measurement of the ALP level is based on the ALP-mediated conversion of p-nitrophenol phosphate (PNPP) to nitrophenol in an alkaline buffer (25). The product nitrophenol exhibits the light absorption at a 405-nm wavelength. In the present study, the ALP levels of human osteoblasts cultured on the 5th day with ^{13}C-enriched testosterone at the concentrations of 0, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l were determined. Following the instructions of the Alkaline Phosphatase Assay kit (TW-Reagent Industrial Co., Ltd.), the cells were lysed in 600 μl lysis buffer and the lysate was centrifuged at 1,000 x g for 20 min at 20˚C. The supernatant and PNPP solution were added to the well of a tissue culture plate and then incubated at 37˚C for 1 h. After the stop solution provided with the Alkaline Phosphatase Assay kit was added, the light absorbance was measured at wavelength of 405 nm using a microplate reader (Synergy LX, Bio-Tek Instruments, Inc.). The values of the ALP level (U/l) were recorded. The test was repeated six times, and the average value was recorded. The data were normalized to the control.

Measurement of OC secretion levels of osteoblasts. In order to investigate the isotope effect of ^{13}C-enriched testosterone on the OC secretion of osteoblasts, the OC level of osteoblasts was examined. OC, an osteoblast-specific secreted protein, is synthesized by osteoblasts during bone formation (26). It plays key roles in both the biological and mechanical functions of bone (13,27). As a biochemical marker of osteoblast activity, the OC level reflects the rate of bone formation (28). In the present study, the OC levels of human osteoblasts cultured on the 5th day with ^{13}C-enriched testosterone at the concentrations of 0, 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l were determined. Following the instructions of the ELISA kit, the OC levels in the supernatant of the culture medium were analyzed using an OC ELISA kit (cat. no. RAB1073-1KT; Sigma-Aldrich; Merck KGaA). The measurement of the OC level (μg/l) was repeated six times, and the average value was recorded. The data were normalized to the control.

Statistics analysis. Data were analyzed using IBM SPSS Statistics v26 software (IBM Corp.). The distribution of the variables was examined using the Shapiro-Wilk test, and the variance was determined using Levene's test. When the distribution was found to be parametric, the differences were assessed using one-way ANOVA with the Bonferroni post hoc test. P-values <0.05 were considered to indicate statistically significant differences. The numerical variables are presented as the mean ± SD (n=6 repeated experiments).

Results

Cell proliferative activity. The proliferative activities of the human osteoblasts, human primary aortic endothelial cells and human umbilical vein endothelial cells treated with testosterone and ^{13}C-enriched testosterone were analyzed. The measured OD values were normalized and plotted (Fig. 2). The morphology of the cells treated with testosterone and ^{13}C-enriched testosterone at the concentration of 10^{-5} mol/l is illustrated in Fig. 3.

Human osteoblasts. Testosterone promoted the proliferation of human osteoblasts at the concentrations of 10^{-10} ($P<0.01$) and 10^{-8} mol/l ($P<0.05$). Following treatment with supraphysiological concentrations (10^{-5} mol/l) of testosterone, the human osteoblasts exhibited a decreasing trend in proliferative activity (Fig. 2A).

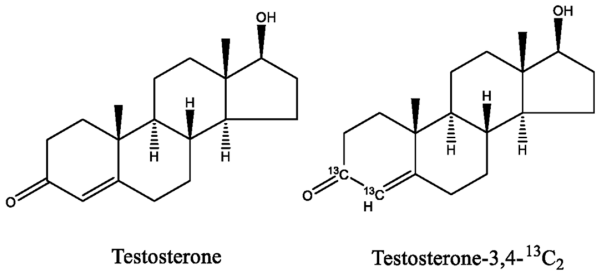

Figure 1. Chemical structures of testosterone and testosterone-3,4-$^{13}C_2$. ^{13}C, carbon-13.

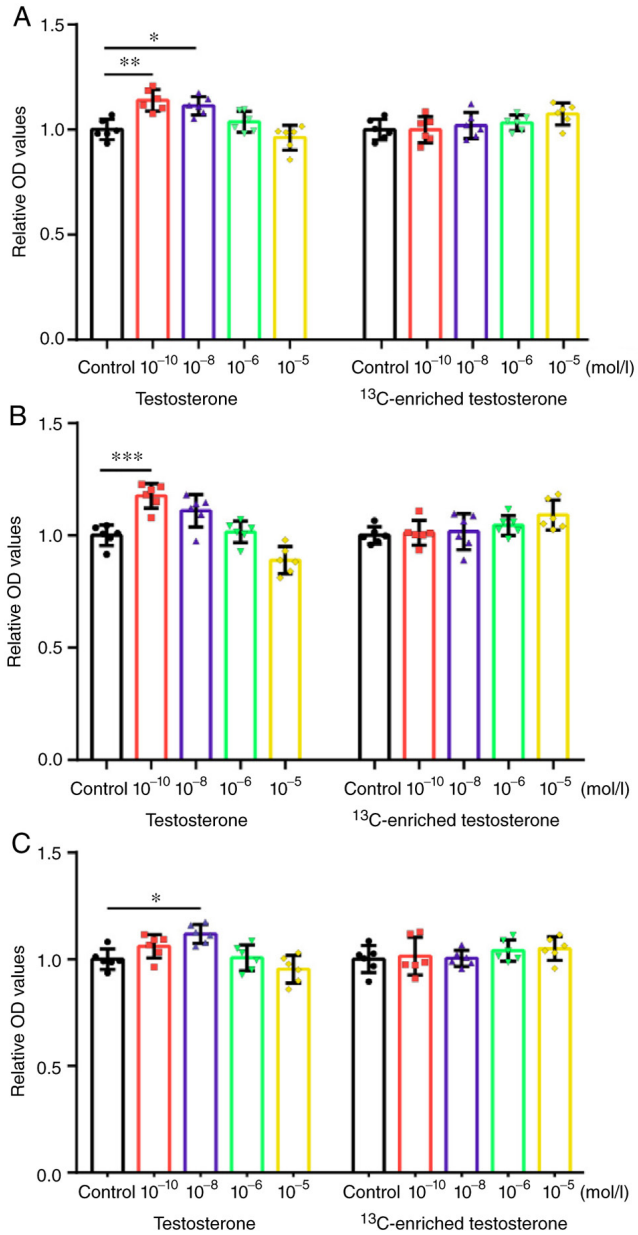

Figure 2. Effects of testosterone and ^{13}C-enriched testosterone on human cells. (A) Effects of testosterone and ^{13}C-enriched testosterone on human osteoblasts. (B) Effects of testosterone and ^{13}C-enriched testosterone on human primary aortic endothelial cells. (C) Effects of testosterone and ^{13}C-enriched testosterone on human umbilical vein endothelial cells. The cells were treated with testosterone and ^{13}C-enriched testosterone at various concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l). Data represent the mean ± SD (n=6 repeated experiments). *P<0.05, **P<0.01 and ***P<0.001, significant difference vs. control (no treatment). ^{13}C, carbon-13.

^{13}C-enriched testosterone did not promote the proliferation of human osteoblasts at the concentrations of 10^{-10}-10^{-6} mol/l. Among these concentration groups, there were no marked differences in the effects of ^{13}C-enriched testosterone, which suggested that the effect had no association with the concentration at the level of 10^{-10}-10^{-6} mol/l. It was noted that the human osteoblasts exhibited an increasing trend in proliferative activity when treated with supraphysiological concentrations (10^{-5} mol/l) of ^{13}C-enriched testosterone, although no significant differences were found (Fig. 2A).

Human primary aortic endothelial cells. Testosterone significantly promoted the proliferation of human primary aortic endothelial cells at the concentration of 10^{-10} mol/l (P<0.001). Following treatment with supraphysiological concentrations (10^{-5} mol/l) of testosterone, the human primary aortic endothelial cells exhibited a decreasing trend in proliferative activity (Fig. 2B).

^{13}C-enriched testosterone did not promote the proliferation of human primary aortic endothelial cells at the concentrations of 10^{-10}-10^{-6} mol/l. Among these concentration groups, there were no differences in the effects of ^{13}C-enriched testosterone, which indicated that the effect had no association with the concentration at the level of 10^{-10}-10^{-6} mol/l. It was noted that the human primary aortic endothelial cells exhibited an increasing trend in proliferative activity when treated with a high concentration (10^{-5} mol/l) of ^{13}C-enriched testosterone, although no significant differences were found (Fig. 2B).

Human umbilical vein endothelial cells. Testosterone significantly promoted the proliferation of human umbilical vein endothelial cells at the concentration of 10^{-8} mol/l (P<0.05). Following treatment with a high concentration (10^{-5} mol/l) of testosterone, the human umbilical vein endothelial cells exhibited a decreasing trend in proliferative activity (Fig. 2C).

^{13}C-enriched testosterone did not increase the proliferation of human vein endothelial cells at the concentrations of 10^{-10}-10^{-5} mol/l. Among these concentration groups, there were no significant differences in the effects of ^{13}C-enriched testosterone, which indicated that the effect had no association with the concentration at the level of 10^{-10}-10^{-5} mol/l (Fig. 2C).

ALP level of human osteoblasts. Compared with the control group, ^{13}C-enriched testosterone did not enhance the ALP level of human osteoblasts at the concentrations of 10^{-10} and 10^{-8} mol/l. The human osteoblasts exhibited an increasing trend in ALP levels when treated with a high concentration (10^{-6} and 10^{-5} mol/l) of ^{13}C-enriched testosterone, although no significant differences were found (Fig. 4).

Osteocalcin secretion of human osteoblasts. Compared with the control group, ^{13}C-enriched testosterone significantly enhanced the OC level in human osteoblasts at the concentrations of 10^{-6} (P<0.05) and 10^{-5} mol/l (P<0.001) (Fig. 5).

Discussion

Among the biogenic elements composing 96% of the human body (29), carbon is a vital element and composes the backbone of biological macromolecules. As biochemical reactions are often accompanied by the cleavage or formation of

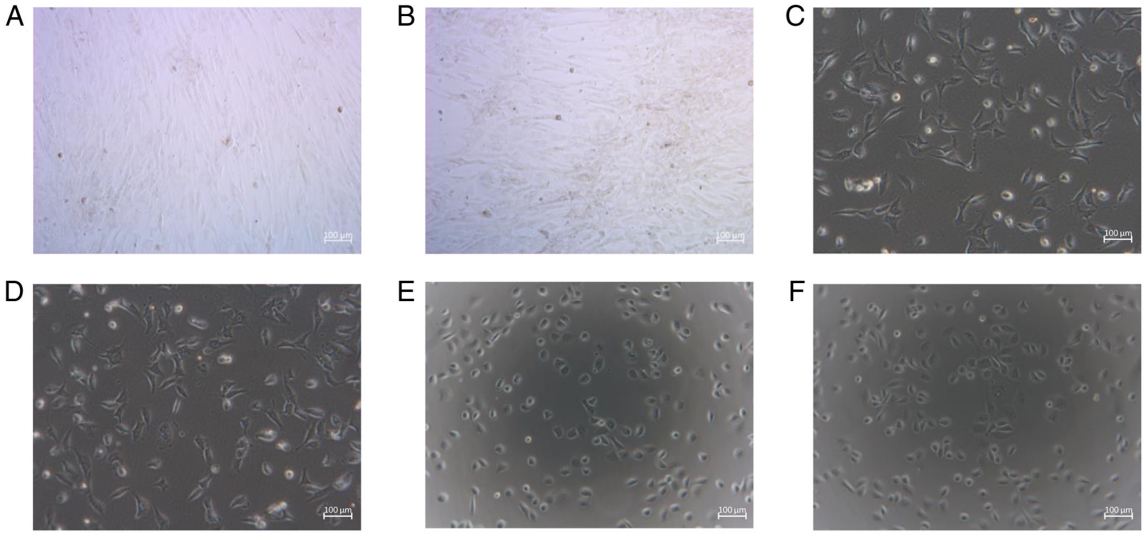

Figure 3. Morphology of cells treated with testosterone and ^{13}C-enriched testosterone at the concentration of 10^{-5} mol/l. (A) Human osteoblasts cells treated with testosterone (magnification, x100). (B) Human osteoblasts cells treated with ^{13}C-enriched testosterone (magnification, x100). (C) Human primary aortic endothelial cells treated with testosterone (magnification, x100). (D) Human primary aortic endothelial cells treated with ^{13}C-enriched testosterone (magnification, x100). (E) Human umbilical vein endothelial cells treated with testosterone (magnification, x100). (F) Human umbilical vein endothelial cells treated with ^{13}C-enriched testosterone (magnification, x100). Scale bar, 100 μm. ^{13}C, carbon-13.

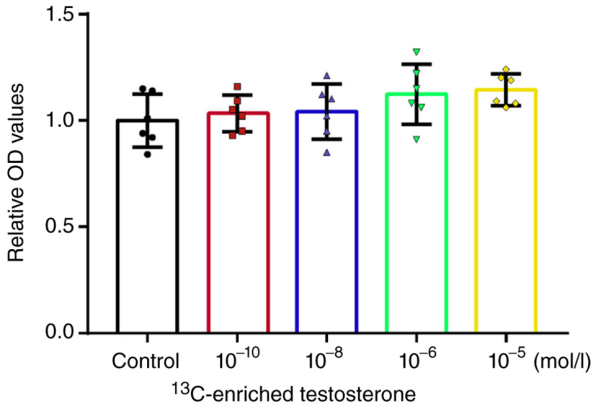

Figure 4. Effects of ^{13}C-enriched testosterone on the alkaline phosphatase level of human osteoblasts. The cells were treated with ^{13}C-enriched testosterone at various concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l). Data represent the mean ± SD (n=6 repeated experiments). ^{13}C, carbon-13.

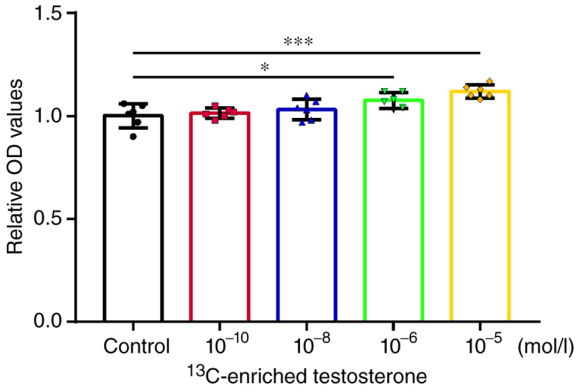

Figure 5. Effects of ^{13}C-enriched testosterone on osteocalcin secretion of human osteoblasts. The cells were treated with ^{13}C-enriched testosterone at various concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/l). Data represent the mean ± SD (n=6 repeated experiments). *P<0.05 and ***P<0.001, significant difference vs. control (no treatment). ^{13}C, carbon-13.

carbon-carbon bonds, the change in carbon isotope composition potentially influences the biochemical reaction rate and exerts a biological isotope effect. Even though the carbon isotope effect is crucial to biological research and medicine, related studies are very limited (30). The present study demonstrated that there was a difference in the biological effect between ^{13}C-enriched testosterone and testosterone. In future studies, the authors aim to elucidate the mechanisms responsible for this phenomenon by investigating other biomarkers secreted by cells and the related signaling pathways.

The growth rate is arguably the most profound phenotypic parameter that defines the existence of an organism. It integrates multiple aspects of the physiological state of a cell, and is often associated with how cells respond to drugs (31). Previous studies have established that testosterone exerts effects on cell proliferation in a concentration-dependent manner (13,15,20,21), which was supported by the findings of the present study. Of note, the effects of ^{13}C-enriched testosterone were concentration-independent at 10^{-10}-10^{-6} mol/l concentrations. This finding suggested that ^{13}C enrichment in a drug may alter the pharmacological properties of the drug.

The concentration gradient used in the present study was designed according to the physiological levels of testosterone, which was beneficial to observe the effects in a physiological and supraphysiological state. However, higher gradients of testosterone concentration need to be applied in future studies to supplement the current findings, since a higher gradient may result in a more significant difference in the effects between testosterone and ^{13}C-enriched testosterone.

It is considered that the enrichment of a heavy isotope attenuates the biochemical reaction rate and delays the growth of an organism due to the kinetic isotope effect (2,32-34). This view was supported by the findings of the present study using ^{13}C-enriched testosterone at physiological concentrations;

however, this view was challenged by the results obtained when using supraphysiological concentrations. ^{13}C-enriched testosterone was found to promote cell proliferation at a high concentration (10^{-5} mol/l). Furthermore, ^{13}C-enriched testosterone enhanced the OC secretion of human osteoblasts at high concentrations (10^{-6} and 10^{-5} mol/l). These findings demonstrated the polytropic characteristics of the biological isotope effects, which should be taken into account in stable isotope-based research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW and WY contributed to the conception of the study. MZ and TZ performed the experiments. XW, MZ and WY performed the data analyses and wrote the manuscript. XW, MZ and WY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Iotti S, Raff L and Sabatini A: Chemical and biochemical thermodynamics: Is it time for a reunification? Biophys Chem 221: 49-57, 2017.
2. Andriukonis E and Gorokhova E: Kinetic 15N-isotope effects on algal growth. Sci Rep 7: 44181, 2017.
3. Buchachenko AL: Mass-independent isotope effects. J Phys Chem B 117: 2231-2238, 2013.
4. Basov A, Fedulova L, Vasilevskaya E and Dzhimak S: Possible mechanisms of biological effects observed in living systems during ^{2}H/^{1}H isotope fractionation and deuterium interactions with other biogenic isotopes. Molecules 24: 4101, 2019.
5. Rodin S, Rebellato P, Lundin A and Zubarev RA: Isotopic resonance at 370 ppm deuterium negatively affects kinetics of luciferin oxidation by luciferase. Sci Rep 8: 16249, 2018.
6. Kozin S, Skrebitsky V, Kondratenko R, Kravtsov A, Butina E, Moiseev A, Malyshko V, Baryshev M, Elkina A and Dzhimak S: Electrophysiological activity and survival rate of rats nervous tissue cells depends on D/H isotopic composition of medium. Molecules 26: 2036, 2021.
7. Li X and Snyder MP: Yeast longevity promoted by reversing aging-associated decline in heavy isotope content. NPJ Aging Mech Dis 2: 16004, 2016.
8. Li X and Snyder MP: Can heavy isotopes increase lifespan? Studies of relative abundance in various organisms reveal chemical perspectives on aging. Bioessays 38: 1093-1101, 2016.
9. Hill S, Hirano K, Shmanai VV, Marbois BN, Vidovic D, Bekish AV, Kay B, Tse V, Fine J, Clarke CF and Shchepinov MS: Isotope-reinforced polyunsaturated fatty acids protect yeast cells from oxidative stress. Free Radic Biol Med 50: 130-138, 2011.
10. Zlatskiy IA, Zlatska AV, Antipova NV, Dolenko SA, Gordiienko IM, Gubar OS, Vasyliev RG, Zubov DA, Novikova SN and Syroeshkin AV: Comparative analysis of the different dyes' potential to assess human normal and cancer cell viability in vitro under different D/H ratios in a culture medium. ScientificWorldJournal 2020: 2373021, 2020.
11. Xie XS and Zubarev RA: Effects of low-level deuterium enrichment on bacterial growth. PLoS One 9: e102071, 2014.
12. Andreyev AY, Tsui HS, Milne GL, Shmanai VV, Bekish AV, Fomich MA, Pham MN, Nong Y, Murphy AN, Clarke CF and Shchepinov MS: Isotope-reinforced polyunsaturated fatty acids protect mitochondria from oxidative stress. Free Radic Biol Med 82: 63-72, 2015.
13. Wu XC and Zhang MQ: Effects of androgen and progestin on the proliferation and differentiation of osteoblasts. Exp Ther Med 16: 4722-4728, 2018.
14. Vandewalle S, Van Caenegem E, Craen M, Taes Y, Kaufman JM and T'Sjoen G: Growth, sexual and bone development in a boy with bilateral anorchia under testosterone treatment guided by the development of his monozygotic twin. J Pediatr Endocrinol Metab 31: 361-367, 2018.
15. Campelo AE, Cutini PH and Massheimer VL: Cellular actions of testosterone in vascular cells: Mechanism independent of aromatization to estradiol. Steroids 77: 1033-1040, 2012.
16. Lorigo M, Mariana M, Lemos MC and Cairrao E: Vascular mechanisms of testosterone: The non-genomic point of view. J Steroid Biochem Mol Biol 196: 105496, 2020.
17. Littleton-Kearney M and Hurn PD: Testosterone as a modulator of vascular behavior. Biol Res Nurs 5: 276-285, 2004.
18. Mohamad NV, Soelaiman IN and Chin KY: A concise review of testosterone and bone health. Clin Interv Aging 11: 1317-1324, 2016.
19. Russell PK, Clarke MV, Cheong K, Anderson PH, Morris HA, Wiren KM, Zajac JD and Davey RA: Androgen receptor action in osteoblasts in male mice is dependent on their stage of maturation. J Bone Miner Res 30: 809-823, 2015.
20. Cai J, Hong Y, Weng C, Tan C, Imperato-McGinley J and Zhu YS: Androgen stimulates endothelial cell proliferation via an androgen receptor/VEGF/cyclin A-mediated mechanism. Am J Physiol Heart Circ Physiol 300: H1210-H1221, 2011.
21. Ling S, Dai A, Williams MRI, Myles K, Dilley RJ, Komesaroff PA and Sudhir K: Testosterone (T) enhances apoptosis-related damage in human vascular endothelial cells. Endocrinology 143: 1119-1125, 2002.
22. Clark RV, Wald JA, Swerdloff RS, Wang C, Wu FCW, Bowers LD and Matsumoto AM: Large divergence in testosterone concentrations between men and women: Frame of reference for elite athletes in sex-specific competition in sports, a narrative review. Clin Endocrinol (Oxf) 90: 15-22, 2019.
23. Lee W, Eo SR, Choi JH, Kim YM, Nam MH and Seo YK: The osteogenic differentiation of human dental pulp stem cells through G0/G1 arrest and the p-ERK/Runx-2 pathway by sonic vibration. Int J Mol Sci 22: 10167, 2021.
24. Zhang Y, Sun Y, Liu J, Han Y and Yan J: MicroRNA-346-5p regulates differentiation of bone marrow-derived mesenchymal stem cells by inhibiting transmembrane protein 9. Biomed Res Int 2020: 8822232, 2020.
25. Sun J, Zhao J, Bao X, Wang Q and Yang X: Alkaline phosphatase assay based on the chromogenic interaction of diethanolamine with 4-aminophenol. Anal Chem 90: 6339-6345, 2018.
26. Mizokami A, Kawakubo-Yasukochi T and Hirata M: Osteocalcin and its endocrine functions. Biochem Pharmacol 132: 1-8, 2017.
27. Bailey S, Karsenty G, Gundberg C and Vashishth D: Osteocalcin and osteopontin influence bone morphology and mechanical properties. Ann NY Acad Sci 1409: 79-84, 2017.
28. Wada S, Fukawa T and Kamiya S: Osteocalcin and bone. Clin Calcium 17: 1673-1677, 2007.
29. Xie X and Zubarev RA: Isotopic resonance hypothesis: Experimental verification by Escherichia coli growth measurements. Sci Rep 5: 9215, 2015.

30. Gregg CT, Hutson JY, Prine JR, Ott DG and Furchner JE: Substantial replacement of mammalian body carbon with carbon-13. Life Sci 13: 775-782, 1973.
31. Kopf SH, Sessions AL, Cowley ES, Reyes C, Van Sambeek L, Hu Y, Orphan VJ, Kato R and Newman DK: Trace incorporation of heavy water reveals slow and heterogeneous pathogen growth rates in cystic fibrosis sputum. Proc Natl Acad Sci USA 113: E110-E116, 2016.
32. Gorokhova E: Shifts in rotifer life history in response to stable isotope enrichment: Testing theories of isotope effects on organismal growth. R Soc Open Sci 4: 160810, 2017.
33. Xie X and Zubarev RA: On the effect of planetary stable isotope compositions on growth and survival of terrestrial organisms. PLoS One 12: e0169296, 2017.
34. Pomytkin IA and Kolesova OE: Relationship between natural concentration of heavy water isotopologs and rate of H2O2 generation by mitochondria. Bull Exp Biol Med 142: 570-572, 2006..