Observations on biological effects of carbon-13, based on the effects of $^{13}$C-testosterone on growth of human osteoblasts, human aortic endothelial cells, and human umbilical vein endothelial cells in vitro

Mengqi Zhang1 · Wenning Yang2 · Xinchen Wu3 · Tengfei Zhang3
1 Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, People’s Republic of China
2 School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 102488, People’s Republic of China
3 Sanbo brain hospital, Capital Medical University, Beijing 100093, People’s Republic of China
Corresponding author: Wenning Yang, e-mail address: Yangwn0523@163.com; Xinchen Wu, e-mail address: xinchenwu1990@163.com

Abstract
Despite the increasing knowledge of biological isotope effect, comprehensive understanding of heavy isotope effect in the biological contexts has remained far less than expectation. The present study investigated the carbon isotope effect of $^{13}$C enriched testosterone on human cells. It was among the rare studies on carbon isotope effect of bioactive compound. Human osteoblasts, human aortic endothelial cells, and human umbilical vein endothelial cells were cultured in vitro and treated with testosterone and $^{13}$C enriched testosterone ($^{13}$C/$^{12}$C: 6.7%). The impacts of physiological to pharmacological concentrations ($10^{-10}$-$10^{-5}$mol/L) of the bioactive compound were taken into account. The cell proliferation activities were measured using MTS assay. The levels of alkaline phosphatase and osteocalcin in osteoblasts were tested. Our results established that $^{13}$C enriched testosterone exhibited different biological effects from testosterone. At the concentrations of $10^{-10}$mol/L and $10^{-5}$mol/L, there were significant differences in prompting cell proliferation between testosterone and $^{13}$C enriched testosterone. At physiological concentrations, testosterone prompted proliferations of the three kinds of cells; whereas, $^{13}$C enriched testosterone did not prompt the cell proliferation, and its effects were not concentration dependent. At supraphysiological concentration ($10^{-5}$mol/L), testosterone had the trend of inhibiting cell growth; whereas, $^{13}$C enriched testosterone had the trend of prompting cell growth. $^{13}$C enriched testosterone significantly enhanced osteocalcin secretion in human osteoblasts at supraphysiological concentration. These findings challenged the common view of growth retardation effect of heavy isotope, which imply that biological isotope effects are worthy of further study. The potential applications of $^{13}$C enriched compound were discussed.

Keywords Stable isotope, Carbon isotope effect, Human osteoblasts, Human aortic endothelial cells, Human umbilical vein endothelial cells, Testosterone

Declarations
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Conflicts of interest/Competing interests
The authors declare that they have no conflict of interest.

Availability of data and material
All data generated during this study are included in supplementary information files of this published article.

Code availability
All codes that were written for analyses in this study are included in supplementary information files of this published article.

Authors' contributions
Xinchen Wu, Wenning Yang contributed to the conception of the study;
Mengqi Zhang, Tengfei Zhang performed the experiment;
Xinchen Wu, Tengfei Zhang contributed significantly to analysis and manuscript preparation;
Mengqi Zhang, Wenning Yang performed the data analyses and wrote the manuscript.

Ethics approval
'Ethics approval' is not applicable to this study.

Consent to participate
'Consent to participate' is not applicable to this study.

Consent for publication
Written informed consent for publication was obtained from all participants.
Introduction

Biogenic elements (such as C, H, O, and N) constitute organism in the form of stable isotopes with their respective isotope compositions. Large deviation from the intrinsic isotope signature of organism may influence the growth and metabolism of organism, provoking biological isotope effect.

Previous studies have demonstrated the detrimental impacts of heavy isotope on organism [1,2]. It was showed that ingestion of 30-50% D2O impaired hematopoiesis and lowered formation of platelets, neutrophils, and lymphocytes in the mouse [3]. One study with multiple heavy-isotope substitutions presented the abnormal changes in cell size and in the quantity and distribution of cellular components in alga [4]. On the other hand, some investigations have proved the beneficial effect of deuterium uptake, such as, promotion of longevity and health in several organisms [5] and protection of yeast cells from oxidative stress in yeast [6]. These findings are not contradictory, but because of different conditions in study, such as, biogenic element in question, isotope enrichment level, species of organism, and physiological properties observed.

The isotope enrichment level influences the isotope effect on biological system. One assay of bacterial growth found that uptake of more than 0.5% deuterium in water resulted in adverse effects, but ultralow deuterium enrichment (≤0.25%) showed signs of the opposite trend [7]. Another investigation with polyunsaturated fatty acid (PUFA) in yeast determined 20-50% of deuterated-PUFA in the total pool of PUFA as the enrichment levels of mitochondria protection [8]. In some literature, the levels of heavy isotope enrichment were classified as high level (>50%) and low level (<10%) [7].

Despite the increasing knowledge of biological isotope effect, comprehensive understanding of heavy isotope function in the biological contexts has remained far less than expectation. The following aspects are important for the theory and application study on biological isotope effect.

1) There have been fewer studies on the effect of other heavy isotope than deuterium. Carbon isotope effect on organism is rarely studied.

2) Compared with other organism, fewer isotope effects on human system have been investigated.

3) The isotopically modified water, diet, and culture medium were used in most of the studies to introduce heavy isotopes into organism. Although this approach can be used to observe the impact of isotope substitution within the whole body, it has its own limitations. It is not conducive to study the isotope effect of a bioactive compound. Study with bioactive compound can provide more information at molecular level and facilitate to develop isotopically modified drugs. Bioactive compound acts mainly on the target tissue, while diet acts on the whole body. Diet-induced isotope effect including potential toxicity to organism is systemic. Little research on isotope effect of bioactive compound has been reported.

4) It is well recognized that a number of bioactive substances, such as steroid hormones, perform normal functions only at the physiological concentrations. Deficiency or excess of the substance may produce negative effects on health. As for these substances, both isotope enrichment level and experimental concentration should be taken into account in order to understanding biology isotope effect comprehensively.

The issues mentioned above were of the interests of our research. Testosterone, a bioactive compound, was isotopically modified to investigate the carbon isotope effect on human cells. The androgen is involved in the regulation of a number of physiological processes including bone development [9,10] and vascular behavior modulation [11-13]. Osteoblasts and vascular endothelial cells express androgen receptor and are targets for androgen action [14-16]. Several studies have established that testosterone produces the concentration-dependent impacts on cell proliferations of
human osteoblasts [9], human aortic endothelial cells [11,16], and human umbilical vein endothelial cells [17]. The present study analyzed the isotope effect of carbon-13 (\(^{13}\)C) enriched testosterone on growth of the three kinds of human cells at various concentrations. It was among the rare studies with regard to carbon isotope effect of bioactive compound on human cells.

The aim of the present study was to investigate the carbon isotope effect of \(^{13}\)C enriched testosterone (\(^{13}\)C/\(^{12}\)C: 6.7\%) on growth of human osteoblasts, human aortic endothelial cells, and human umbilical vein endothelial cells. The impacts of physiological concentrations (\(10^{-10}\)mol/L and \(10^{-8}\)mol/L) [18] and supraphysiological concentrations (\(10^{2}\)mol/L and \(10^{3}\)mol/L) were also investigated with in vitro model.

**Materials and methods**

**Materials and reagents**

Human osteoblasts, human primary aortic endothelial cells, and human umbilical vein endothelial cells were obtained from ScienCell Research Laboratories (Shanghai, China), Cell Biologics (Shanghai, China), and ThermoFisher Scientific (China), respectively. Ascorbic acid, glycerol-2-phosphate, and dexamethasone were purchased from Sigma-Aldrich (China). Dulbecco's modified Eagle's medium (DMEM) with low glucose, penicillin-streptomycin, trypsin-ethylenediaminetraacetic acid (EDTA), fetal bovine serum (FBS), and phosphate buffered saline (PBS) were obtained from ThermoFisher Scientific (China). Testosterone and testosterone-3,4,\(^{13}\)C\(_2\) were from First Standard Company (China).

**Preparation of \(^{13}\)C enriched testosterone**

Testosterone and testosterone-3,4,\(^{13}\)C\(_2\) were dissolved in ethanol respectively and then mixed together with the 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\(_2\) were displayed 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\(^{\circ}\)C. The cells were dissociated with trypsin-EDTA and seeded in 96 well tissue culture plates at the density of \(1\times10^4\) cells/well, the culture media was changed to the media made with low glucose DMEM, 10% FBS, 1% penicillin-streptomycin, 50mg/mL ascorbic acid, 0.01 mol/L glycerol-2-phosphate, and 100nmol/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 \(10^{2}\)mol/L, respectively. Non-drug group (0mol/L) was accompanied as blank control.

**Measurement of cell proliferation activity**

The proliferation activities of human osteoblasts, human aortic endothelial cells, and human umbilical vein endothelial cells were determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (MTS cell proliferation colorimetric assay kit, Amyjet Scientific, China) when the cells were cultured for 48h. Following the manufacturer's instruction, the MTS and phenazine methosulfate (PMS) solution were mixed (MTS:PMS=20:1). The culture medium and the mixed solution of MTS and PMS
(phenazine methosulfate) were added to the test well of 96-well plate and were incubated at 37℃ for 2h. The light absorbance of the formazan product was measured at 490 nm wavelength with a spectrophotometer (BIO-TEK, USA). The measurement for each concentration was repeated six times, and the average value of optical density (OD) was recorded.

Test of alkaline phosphatase level of osteoblasts

In order to further investigate the isotope effect of $^{13}$C enriched testosterone on the osteoblasts, alkaline phosphatase level in osteoblasts was tested. Alkaline phosphatase (ALP) elevation serves as an osteogenic differentiation marker. The test of alkaline phosphatase level is based on ALP-mediated conversion of p-nitrophenol phosphate (PNPP) to nitrophenol in an alkaline buffer. The product nitrophenol exhibits the light absorption at 405 nm wavelength. In the current study, alkaline phosphatase levels of the human osteoblasts cultured at 5th day with $^{13}$C enriched testosterone at the concentrations of 0, $10^{-10}$, $10^{-8}$, $10^{-6}$, and $10^{-5}$mol/L were tested. Following the instruction of Alkaline Phosphatase Assay kit (TW-Reageng Industrial Co., Ltd. Shanghai, China), the cells were lysed in 600 μl lysis buffer and the lysate was centrifuged. The supernatant and PNPP solution were added to the well of a tissue culture plate and then incubated at 37℃ for 1h. After the stop solution was added, the light absorbance was measured at wavelength of 405 nm. The values of ALP level (U/L) were recorded. The test was repeated six times, and the average value was recorded. The data was normalized by the control.

Test of osteocalcin secretion level of osteoblasts

In order to investigate the isotope effect of $^{13}$C enriched testosterone on osteocalcin (OC) secretion in osteoblasts, OC level of the osteoblasts was tested. OC, an osteoblast-specific secreted protein, is synthesized by osteoblasts during bone formation [19]. It plays key roles in both the biological and mechanical functions of bone [9,20]. As a biochemical marker of osteoblast activity, OC level reflects the rate of bone formation [21]. In the current study, the OC levels of the human osteoblasts cultured at 5th day with $^{13}$C enriched testosterone at the concentrations of 0, $10^{-10}$, $10^{-8}$, $10^{-6}$, and $10^{-5}$mol/L were tested. Following the instruction of ELISA kit, the OC in supernatant of the culture medium was analyzed using OC ELISA kit (sigma RAB1073-1KT). The test of OC level (μg/L) was repeated six times, and the average value was recorded. The data was normalized by the control.

Statistics

Data were analyzed with SPSS software. Significant differences were assessed with ANOVA and Post-hoc Test. P<0.05 were considered significant.

Results and Discussion

Cell proliferation activity

The proliferation activities of human osteoblasts, human 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 in Fig. 2-4.
testosterone, human osteoblasts showed a decreasing trend of proliferation activity (Fig. 2).

$^{13}$C enriched testosterone did not prompt the proliferation of human osteoblasts at 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 implied that the effect had no relationship with the concentration at the level of $10^{-10}$-$10^{-6}$mol/L. It was noted that human osteoblasts showed an increasing trend of proliferation activity when treated with supraphysiological concentration ($10^{-5}$mol/L) of $^{13}$C enriched testosterone (Fig. 2).

There were significant differences in proliferation activity between treatment of testosterone and $^{13}$C enriched testosterone at the concentration of $10^{-10}$mol/L ($P<0.01$) and $10^{-5}$mol/L ($P<0.05$), respectively (Fig. 2).

**Human aortic endothelial cells**

Testosterone significantly prompted the proliferation of human aortic endothelial cells at the concentration of $10^{-10}$mol/L ($P<0.001$). Treated with supraphysiological concentration ($10^{-5}$mol/L) of testosterone, human aortic endothelial cells showed a decreasing trend of proliferation activity (Fig. 3).

$^{13}$C enriched testosterone did not prompt the proliferation of human 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 implied that the effect had no relationship with the concentration at the level of $10^{-10}$-$10^{-6}$mol/L. It was noted that human aortic endothelial cells showed an increasing trend of proliferation activity when treated with high concentration ($10^{-5}$mol/L) of $^{13}$C enriched testosterone (Fig. 3).

There were significant differences in proliferation activity of human aortic endothelial cells between treatment of testosterone and $^{13}$C enriched testosterone at the concentrations of $10^{-10}$mol/L ($P<0.001$) and $10^{-5}$mol/L ($P<0.0001$), respectively (Fig. 3).

**Human umbilical vein endothelial cells**

Testosterone prompted the proliferation of human umbilical vein endothelial cells at the concentration of $10^{-8}$mol/L ($P<0.05$). Treated with high concentration ($10^{-5}$mol/L) of testosterone, the human umbilical vein endothelial cells showed a decreasing trend of proliferation activity (Fig. 4).

$^{13}$C enriched testosterone did not prompt 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 implied that the effect had no relationship with the concentration at the level of $10^{-10}$-$10^{-5}$mol/L (Fig. 4).

There were no differences in proliferation activity of human umbilical vein endothelial cells between treatment of testosterone and $^{13}$C enriched testosterone at the concentrations of $10^{-10}$-$10^{-5}$mol/L.

**ALP level of human osteoblasts**

Compared with control group, $^{13}$C enriched testosterone did not enhance ALP level of human osteoblasts at the concentrations of $10^{-10}$mol/L and $10^{-8}$mol/L. The human osteoblasts showed increasing trend of ALP level when treated with high concentration ($10^{-6}$mol/L and $10^{-5}$mol/L) of $^{13}$C enriched testosterone (Fig. 5).

**Osteocalcin secretion of human osteoblasts**

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

**Discussion**

The present research contributed novel contents to the biology isotope effect of carbon. The elements C, H, O and N compose over 98% of the total living mass in the Earth and account for 96% of human body weight [22]. Biological effects of heavy isotopes of the elements have been constantly recognized. Deuterium has been investigated more than other heavy stable isotopes, which is partly due to the consideration that deuterium with twice the mass of a hydrogen atom exhibits more remarkable isotope effect. However, carbon is a vital element of biological system and forms the skeleton of biological macromolecules. Maintaining physiological activities, numerous biochemical reactions occur in organism with carbon-carbon bond formation and cleavage. The change in carbon isotope composition could influence the biochemical reaction rate and produce biology isotope effect. Therefore, carbon isotope effect on human and other organisms should be intensively studied for the potential application in biology and medicine.

Our study proved that $^{13}\text{C}$ enriched testosterone at 6.7% of $^{13}\text{C}/^{12}\text{C}$ had different biological effects from testosterone. We assumed that the enrichment of heavy isotope in bioactive compound could weaken biological effect of the compound, which potentially benefit drug development. For example, administration of $^{13}\text{C}$ enriched testosterone would relieve symptoms of hyperandrogenism, such as acne, hirsutism, and hyperinsulinemia [23].

Previous studies established that testosterone had effects on the cell proliferations in a concentration-related manner [9,11,16,17], which was supported by our findings. However, the effects of $^{13}\text{C}$ enriched testosterone were not concentration dependent at the concentration levels of $10^{-10}$–$10^{-6}\text{mol/L}$. This finding implied that enrichment of heavy isotope in drug might change the dose dependence of the drug action. For the drugs whose effective dose and toxic dose are close to each other, enrichment of heavy carbon isotope in the drug might make its effect less sensitive to the change of concentration, which could potentially improve the drug safety.

It has been generally believed that enrichment of heavy isotope slow down the biochemical reaction rate and retard the growth of organism because of kinetic isotope effect [24-28]. This common view was supported by only a part of our findings. It was unexpected that $^{13}\text{C}$ enriched testosterone showed the trends of promoting the cell proliferations at high concentration ($10^{-4}\text{mol/L}$). Furthermore, $^{13}\text{C}$ enriched testosterone enhanced OC secretion of human osteoblasts significantly at high concentrations ($10^{-6}\text{mol/L}$ and $10^{-5}\text{mol/L}$). These findings challenged the common view of heavy isotope enrichment and exhibited the complexity of the biology isotope effect. The underline mechanisms are poorly understood and should be further investigated.

**Conclusions**

The present study substantiated the carbon isotope effect of one bioactive compound. $^{13}\text{C}$ enriched testosterone at 6.7% of $^{13}\text{C}/^{12}\text{C}$ had different biological effects from testosterone. At physiological concentrations, testosterone prompted the cell proliferations; whereas, $^{13}\text{C}$ enriched testosterone did not prompt the cell proliferations and its effects were not concentration dependent. At supraphysiological concentration ($10^{-4}\text{mol/L}$), testosterone had the trend of inhibiting cell growth; whereas, $^{13}\text{C}$ enriched testosterone had the trend of prompting cell growth. $^{13}\text{C}$ enriched testosterone significantly enhanced osteocalcin secretion in human osteoblasts at supraphysiological concentration. These findings imply
that biology isotope effects are worthy of further study to benefit human health.

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Figures

Fig. 1 The chemical structure of testosterone and testosterone-3,4-\(^{13}\)C\(_2\).

![Chemical Structure of Testosterone and Testosterone-3,4-\(^{13}\)C\(_2\)](image)

Fig. 2 The influence of testosterone and \(^{13}\)C enriched testosterone on human osteoblasts. The cells were treated with testosterone and \(^{13}\)C enriched testosterone at different concentrations (10\(^{-10}\), 10\(^{-8}\), 10\(^{-6}\) and 10\(^{-5}\) mol/L, respectively). Data represent means± SEM (n= 6). Significant difference *p < 0.05, **p< 0.01 vs. compared.

![Graph showing influence of testosterone and \(^{13}\)C enriched testosterone on human osteoblasts](image)

Fig. 3 The influence of testosterone and \(^{13}\)C enriched testosterone on human aortic endothelial cells. The cells were treated with testosterone and \(^{13}\)C enriched testosterone at different concentrations (10\(^{-10}\), 10\(^{-8}\), 10\(^{-6}\) and 10\(^{-5}\) mol/L, respectively). Data represent means± SEM (n= 6). Significant difference ***p < 0.001, ****p< 0.0001 vs. compared.

![Graph showing influence of testosterone and \(^{13}\)C enriched testosterone on human aortic endothelial cells](image)
Fig. 4 The influence of testosterone and 13C enriched testosterone on human umbilical vein endothelial cells. The cells were treated with testosterone and 13C enriched testosterone at different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/L, respectively). Data represent means± SEM (n= 6). Significant difference *p < 0.05 vs. compared.

![Graph showing relative OD values for testosterone treatment](image1)

Fig. 5 The influence of 13C enriched testosterone on ALP level of human osteoblasts. The cells were treated with 13C enriched testosterone at different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/L, respectively). Data represent means± SEM (n= 6).

![Graph showing relative OD values for ALP level](image2)

Fig. 6 The influence of 13C enriched testosterone on osteocalcin secretion of human osteoblasts. The cells were treated with 13C enriched testosterone at different concentrations (10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} mol/L, respectively). Data represent means± SEM (n= 6). Significant difference *p<0.05, ***p<0.001 vs. control (treated with same amount of vehicle).
