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# Hyperbaric oxygen treatment increases intestinal stem cell proliferation through mTORc1 signaling in Mus musculus

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# Hyperbaric oxygen treatment increases intestinal stem cell proliferation through mTORc1 signaling in *Mus musculus*

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**Abbreviations:** Hyperbaric oxygen treatment (HBOT), mTOR complex 1 (mTORc1), intestinal stem cells (ISCs), caloric restriction (CR), columnar base cells (CBC), Bromodeoxyuridine (BrdU), phospho-Histone H3 (PHH3), cytochrome c oxidase (COX), citrate synthase (CS), immunofluorescence (IF), immunohistochemistry (IHC)

**Abstract**

**Background:** Hyperbaric oxygen treatment (HBOT) has been used for more than a decade to treat diverse diseases like diabetic foot ulcers and ischemic injuries. More recently, HBOT has been reported to modulate proliferation of neural and intestinal stem cell populations, but the molecular mechanisms underlying these effects are not completely understood.

**Objective:** In this study we aimed to determine HBOT stem cell modulation by evaluating in particular the role of the mTOR complex 1 (mTORc1), a key regulator of cell metabolism that modifies its activity depending on oxygen levels, as a potential mediator of HBOT in murine intestinal stem cells (ISCs).

**Methods:** Mouse were exposed to 10 or 20 HBOT sessions and the proliferation of the ISCs were analyzed by immunofluorescence or immunohistochemistry using the specific ISCs marker, Olfm4. The regulation of HBOT and mTORc1 pathway was analyzed through S6K1 phosphorylation by western-blot and through the inhibition by rapamycin.

**Results:** We discovered that acute HBOT can increase proliferation of ISCs in a synchronous fashion without affecting the animal's oxidative metabolism. Noteworthy, the mTORc1 inhibitor rapamycin also increases the proliferation of ISCs. This effect has been attributed to its capacity to mimic a caloric restriction (CR). Interestingly, the combination of HBOT and rapamycin does not have a synergic effect. Nevertheless, HBOT can recover rapamycin induced mTORc1 inhibition, possibly acting through a competitive modulation on mTORC1.

**Conclusions:** Collectively, our results suggest that HBOT proliferative effect on ISCs is modulated by mTORc1 signaling representing a promising new approach to treat intestinal conditions.

Keywords: Intestinal stem cells, Hyperbaric oxygen treatment, mTORc1, proliferation, rapamycin.

## Introduction

Hyperbaric oxygen treatment (HBOT) consists in administration of oxygen at high pressures. HBOT works under Henry's law that states that the amount of gas dissolved in a liquid is proportional to the partial pressure that the gas applies over such liquid. As a result, the amount of oxygen dissolved in blood increases, which causes a systemic effect by enriching tissues in oxygen through the bloodstream.

HBOT is widely used as an adjuvant treatment to treat diabetic foot ulcers (Kalani et al; 2002), brain injuries (Zhang et al; 2010 and Bennett et al; 2012) and to promote wound healing (Eskes et al; 2011 and Kranke et al; 2015 and Peña-Villalobos et al; 2018). Recent studies have proposed that its effects on wound healing occurs due modulation of HIF-1α (Sunkari et al; 2015) and Vascular Endothelial Growth Factor (VEGF) (Lee et al; 2006) signaling pathways. Nonetheless, the mechanisms are under continuous debate and still far from being fully understood.

In the last decade, studies have focused on stem cells as cellular targets of HBOT, revealing positive effects on proliferation and regeneration on both neural (Zhang et al; 2011) and mesenchymal stem cell populations (Dhar et al; 2012). An interesting niche for studying the effects of HBOT on stem cell proliferation is the intestinal epithelium, the most vigorously self-renewing tissue of adult mammals (Snippert et al; 2010). Within this epithelium, intestinal stem cells (ISCs) reside at the base of the crypts of Lieberkühn, allowing the generation of absorptive or secretory progenitors who migrate toward the tip of the villus (Barker et al; 2014), regenerating the small intestine every 3 days in mice (Totafurno et al; 1987 and Clevers; 2013). The crypts of Lieberkühn have been well studied and characterized in terms of their cellular composition. ISCs are also named columnar base cells (CBC) for their position in the base of the crypts and can be identified by the expression of specific markers like Lgr5 (a membrane protein) or Olfm4 (a cytoplasmatic protein). CBCs give rise to another functionally distinct population of stem cells, called +4 (due to their position in the crypt) or emergency stem cells. The complex nature of ISCs is becoming clear in recent

88 years, revealing a hierarchy between Lgr5+ and +4 under homeostasis and stress responses (De Mey
89 & Freund; 2013 and Basak et al; 2017).

90 The homeostatic renewal in the intestinal epithelium is commanded by oxidative metabolism and
91 guided by the mTOR complex 1 (mTORc1), a macromolecular nutrient- and growth factor-responsive
92 kinase (Yilmaz et al; 2012 and Sampson et al; 2015). The kinase1 is also able to integrate a response
93 to transcription factors (Igarashi et al; 2016) and oxygen (Schieke et al; 2006 and Saxton & Sabatini;
94 2017). mTORc1 signaling involves the activation of many proteins like 4E-BP1 and S6K1 through
95 phosphorylation, regulating cellular anabolic growth and proliferation (Fingar et al; 2002). A plethora
96 of studies have shown that this complex can be inhibited by the administration of rapamycin, an
97 allosteric inhibitor (Benjamin et al; 2011), preventing the formation of the complex and therefore
98 downregulating the signaling pathway (Fingar et al; 2004). Furthermore, rapamycin has been used to
99 mimic the effects of a caloric restriction (CR), resulting in an increase of lifespan in different species
100 like yeast, mice and humans (Cox et al; 2009, Dai et al; 2014 and Wang et al; 2017).

101 The regulatory mechanisms that control ISCs in response to HBOT are just beginning to be explored.
102 Here we analyzed the effects of acute HBOT on the ISCs proliferation rates of *Mus musculus* small
103 intestine and its modulation by mTORc1. First, we established the number of HBOT sessions required
104 to promote a positive effect on ISCs proliferation. Secondly, we sought to understand the complex
105 relation between oxygen tension and proliferation, mediated by the mTOR complex. Our results show
106 that animals treated for 10 days with HBOT present a trend to increase ISCs proliferation. However,
107 animals treated with a daily HBOT session for 20 consecutive days show an increase in proliferation
108 in a synchronous fashion among crypts. Noteworthy, although animals treated with rapamycin also
109 increase the number of ISC proliferating by crypt, mimicking the effects of a CR (Cox et al; 2009 and
110 Dai et al; 2014), a combined treatment with HBOT and rapamycin did not increase ISC proliferation.
111 We speculate that the latter is probably due a competition for mTORc1. Strikingly, we found that
112 HBOT can recover the inhibition of rapamycin over mTORC1. Overall, our findings suggest that

HBOT can replicate the effects of a CR, stimulating proliferation of ISCs. As such, we propose that HBOT could be a potential adjuvant treatment for intestinal injuries and pathologies.

## Methods

*Animals*

Forty-six adult (3 months old) males of the BALB/c strain of *Mus musculus* were obtained from the central animal housing facilities at the Faculty of Sciences. All animal procedures were in accordance with the Chilean legislation and were approved by Institutional Animal Care and Use Committee (CICUA) at the Universidad de Chile.

To analyze the effects of HBOT on ISCs proliferation we generated four groups: Control group for ten days (C10D), HBOT group for ten days (H10D), Control group for twenty days (C20D) and HBOT for twenty days (H20D) (Table I). Each group was formed by five animals, considering no siblings in the same group. They were housed with their siblings but properly identified and kept in a temperature-controlled room, maintained at 25° ± 1 °C in a LD= 12:12 cycle, with food and water *ad libitum*.

In order to explore the relationship of HBOT with mTORC1 signaling we generated another set of 4 experimental groups: 2 Groups without HBOT sessions but injected with rapamycin (Rapa Ctrl) or with DMSO (vehicle of rapamycin) (DMSO Ctrl), and 2 groups treated with HBOT injected with rapamycin (RAPA HBOT) or with DMSO (DMSO HBOT) (Table II). All groups were treated for 20 consecutive days. Each group included five animals, maintained in the same conditions as described above.

Table I: Animal groups generated for analysis of HBOT impact on ISCs proliferation.

| **ISCs proliferation** | | |
|---|---|---|
| | **10 Days** | **20 Days** |
| **Control** | C10D | C20D |
| **HBOT** | H10D | H20D |

Table II: Animal groups generated for the study of the relationship between HBOT, mTORC1 pathway and ISCs proliferation.

| mTOR and ISCs | | |
|---|---|---|
| | **Control** | **HBOT** |
| **DMSO** | DMSO Ctrl | DMSO HBOT |
| **rapamycin** | Rapa Ctrl | Rapa HBOT |

*HBOT sessions*

HBOT was performed in a 19.56 L experimental chamber at 25ºC (Osorio Hermanos & Cia. Ltd., Quillota, Chile). Animals were placed in individual cages, then the remaining air inside the chamber was replaced with 100% $O_2$ while the pressure was increased for 15 minutes until 2.0 ATA (absolute atmospheres) were reached. The latter conditions were maintained for 1.0 h followed by decompression from 2.0 ATA to atmospheric pressure gradually for other 15 min (figure 1A). Therefore, each session took 1.5 h (Dave et al; 2003 and Peña-Villalobos et al; 2018).

*Rapamycin administration*

Rapamycin (Calbiochem, 553210) was dissolved in DMSO (5 mg/125 µl) and diluted in PBS 1X 1:100 v/v. Every other day rapamycin was injected intraperitoneally (2 mg/kg), always at the same hour and before starting the HBOT session.

*Intestinal tissue dissection*

After the last HBOT session animals were injected intraperitoneally with 800 µl of 20 mg/ml BrdU (Sigma-Aldrich). One hour later the animals were sacrificed by cervical dislocation. Immediately the entire digestive tract was dissected on a cooled surface and the intestine contents were gently removed mechanically. Next, the first third of the small intestine (duodenum) was cut longitudinally and used for histological analysis. This segment was fixed in 4% PFA (Sigma-Aldrich) for 2 h at 4°C followed by dehydration overnight (O.N) in 30% sucrose (Merck) solution. Tissues were embedded in OCT

(Tissue-Tek), placed in a disposable vinyl specimen mold (Tissue-Tek Cryomold) and stored at -80°C until further use.

*Immunostaining*

To realize immunofluorescence (IF) and immunohistochemistry (IHC) we followed the exact same protocol of Peña-Villalobos et al. (2018). Briefly, slides of either 14 and 7 µm thickness were prepared with the epitope-unmasking protocol and incubated overnight at 4°C with 1:100 monoclonal mouse Anti-Bromodeoxyuridine Clone Bu20a (BrdU, Dako, M0744) primary antibody or 1:400 monoclonal rabbit Anti-Olfm4 (Cell Signaling, 39141). For IF, we used 1:500 goat anti mouse Alexa 555 (Life technology, A21424) as a secondary antibody to BrdU and 1:500 goat anti rabbit Alexa 488 (Life technology, A11034) as a secondary to Olfm4. DAPI (Invitrogen) was used to label nuclei. For IHC, we used hematoxylin/eosin (H&E) and DAB staining as described in Peña-Villalobos et al. (2018). To analyze the IHC, an optical microscope (Olympus BX51) at 40X and 100X equipped with a digital camera (Moticam 2500) was used whereas for IF a confocal microscope (Zeiss 710) was used. Z-stacks and image analysis was made by ImageJ and FIJI programs. Figures were created by Biorender.com.

*Enzyme Assays*

The duodenum was homogenized in 10 volumes of phosphate buffer 0.1 M with EDTA 0,002 M (pH 7.3) with an Ultra Turrax homogenizer (20,000 rpm) on ice. Samples were sonicated for 45 seconds on ice using an Ultrasonic Processor VCX 130. Cellular debris was removed by centrifugation for 15 min at 12,000 G at 4°C. Then the supernatant was isolated and used for determination of protein concentration by Bradford method.

Citrate Synthase and Cytochrome C Oxidase activity were determined spectrophotometrically according to Peña-Villalobos et al. 2018.

*Western Blot*

Homogenized duodenums, stored at -80°C with proteases and phosphatases inhibitors, were left on ice for 1 h. 50 µg of the sample was loaded on acrylamide gel posterior to Lowry quantification (550 nm; Tecan M200) and then transferred to a nitrocellulose film. The film then was treated with BSA 5% in TBS-T 0,1% for an hour, washed in PBS and incubated for 12 h with anti-phospho p70 S6K1 (Thr389) (1:1000, Cell signaling) or anti-p70 S6K1 (1:1000, Cell signaling) and anti-β actine (1: 5000, Cell Signaling). For protein detection we used Rabbit anti-mouse HRP (1:5000; Cell signaling) to identify p70 S6K1 and p70 S6K1 phosphorylated and Mouse anti-β actine (1:5000, Cell Signaling) with luminol. X-ray films were obtained after 1 h exposition in dark room for S6K1 and S6K1-P, while β actine detection last 10 seconds of exposition.

*Statistical analysis*

The number of positive labeled cells per villus of each crypt were scored by two independent counters. To determinate if HBOT has effects on the proliferation dynamics of ISCs we counted the number of BrdU+ cells in 36 randomly chosen crypts from the first third of the intestine of each individual (n=3 per treatment). For IF, ISCs positive cells were considered only if they expressed the specific marker Olfm4 (Olfm4+ cells). Permutation test was performed in R software, using a permutation test script of 1000 permutation for each analysis. Data from mTOR inhibition by rapamycin and western blot were also analyzed by permutation test. To analyze the distribution of the data we used a Kolmogorov-Smirnov test. As for the first experiment (HBOT for either 10 or 20 days) we considered a Bonferroni correction, having an $\alpha = 0.0125$.

## Results

*HBOT increases ISCs proliferation in a synchronous fashion*

Stem cells *in vivo* reside in a dynamic and specialized microenvironment, the so- called niche. The stem cell niche is influenced by a variety of factors including physical and metabolic parameters. Oxygen tension is known to be an important cellular input modulating stem cell self-renewal and differentiation potential. Thus, many stem cells, including ISCs, respond actively to changes in oxygen tension.

ISCs are located at the bottom of the intestinal crypt and can be identified as Olfm4+ cells, revealing a co-distribution with the BrdU label (figure 1 B-E). To determine the proliferation effects of HBOT on ISCs, we evaluated the number of BrdU+ cells per crypt in all experimental groups. Only small intestines from mice treated with HBOT for 20 days (H20D) show an increment in the number of BrdU+ cells (figure 1 F-J). Interestingly, another cell population, located at near position to the intestinal crypts, also exhibited BrdU staining (figure 1E). Hence, we cannot discard that HBOT has effects on another cell population, besides the Olfm4+ CBC pool.

HBOT increased proliferation of cells within the crypts in a synchronous way. This was determined by calculating the stabilization coefficient (figure 1K) for each group. H20D was the only group that shows clear differences. To evaluate if this synchronization in proliferation was generated by a possible arrest in the cell cycle due to treatment, we analyzed the ratio between BrdU and PHH3 positive cells in intestinal samples from C20D group or H20D group (data not shown). We did not find differences in these ratios. Hence, we conclude that HBOT does not generate this synchronization of proliferation due to a cell cycle arrest in ISCs.

*HBOT has an accumulative effect in time*

The frequency distribution from groups treated with H10D was different from its control C10D despite not showing differences in the numbers of BrdU+ cells per crypts. When we analyzed the

frequency distribution for each group (figure 2), we found that only control groups showed no differences (p= 0.796). Interestingly, the frequency distribution from H10D was different, both from the control groups and H20D, indicating that, despite not showing significant differences in the number of BrdU+ cells per crypt (figure 1J), H10D already generates a change in the proliferation behavior of the ISCs.

*Acute HBOT does not change enzymatic activity in the small intestine*

Intestines from H20D did not show differences in comparison to their control in the enzymatic activity related to oxidative metabolism. We sought to analyze if the increment in the proliferation of the ISCs by HBOT could be explained by an increase in the activity of cytochrome c oxidase (COX) and citrate synthase (CS), both enzymes related with aerobic ATP generation. To this end, we analyzed the activity of these enzymes in the whole duodenum in each experimental group (figure 3A). No difference was found in the activity of both enzymes, when analyzed per gram, total in tissue or total protein between control (C10D and C20D) and both HBOT groups (figure 3B-C). Therefore, HBOT effects must encompass another molecular pathway to account for its effect.

*HBOT modulates proliferation of the ISCs through mTORC1 pathway activation*

To evaluate if HBOT could activate the mTOR pathway, capable of sensing changes in oxygen levels, we generated four groups, combining rapamycin with or without HBOT (see table II). In line with the literature, animals treated with rapamycin showed a mTORc1 pathway suppression as revealed by the lack of phosphorylation on S6K1 (figure 4A; supplementary figure 1). On the other hand, animals injected with rapamycin vehicle (DMSO) showed no inhibition on the phosphorylation status of S6K1, either with or without HBOT. Stunningly, animals that received a combined treatment with rapamycin and HBOT revealed strong phosphorylation on S6K1 (Figure 4B). Therefore, we suggest that HBOT can recover rapamycin inhibition of mTOR pathway signaling.

Acute HBOT can simulate the effects on ISCs proliferation generated by a CR. When we compared the effects on ISCs proliferation of rapamycin (Rapa Ctrl) and HBOT for 20 days (DMSO HBOT), we found that each treatment increases the number of BrdU+ cells per crypt (figure 5 A-E). Nevertheless, animals treated with both HBOT and rapamycin (Rapa HBOT) show no increase in ISC proliferation.

Finally, a careful analysis of frequency distribution of BrdU+ cells per crypt indicated that only 2 comparisons present no differences. Rapa HBOT and DMSO Ctrl groups exhibited no differences (figure 6F) while Rapa Ctrl and DMSO HBOT groups also showed no differences in their distributions (figure 6G). These data, in combination with the results presented in figure 5E, suggest that HBOT has the same effects on ISCs proliferation as a CR generated by rapamycin.

## Discussion

HBOT has been reported to modulate the stem cells proliferative behavior in two well-known adult stem cell niches, in the bone marrow (Geng et al; 2015) and the brain (Wang et al; 2007). In this work, we demonstrate that HBOT also can enhance the proliferation of ISCs in a synchronous fashion. Moreover, we provide findings that this proliferative effect is mediated by mTORc1 signaling.

Nearly 90% of the intestinal epithelium is replaced every 3–4 days by cells newly generated from the crypt epithelium. However, long-lived ISCs harbor in the crypt bottom to replenish the large amount of disposable functional epithelium. ISCs produce rapidly cycling progenitor cells that migrate up the crypt-villus axis and differentiate into mature epithelial cells that are eventually shed off into the lumen. Small intestine partial pressure of oxygen (PO2) values have been reported to be 2–5%, 3–6% and 5–9% O2 for lumen, mucosa and serosa layers, respectively (Espey; 2013).

To explore whether oxygen tension impacts cellular turnover in the small intestine, we used an established HBOT protocol (Peña-Villalobos et al; 2018). In our previous work, rodents were exposed to HBOT conditions in ten consecutive sessions. We did not find a significant effect of HBOT on cell proliferation, as all groups had similar numbers of BrdU+ cells per crypt. But in the present work, increasing the time length of treatment by applying HBOT for 20 consecutive days lead to a significant cell proliferation rise within the crypts. The effect of HBOT is clearly observable on day 20 but had an accumulative effect that was already evidenced since day 10. The analysis of the frequency distribution of BrdU+ positive cells from C10D with H10D groups indicates that despite showing no differences in the number of BrdU+ cells per crypts (figure 1J), there are differences in the frequency of proliferative cells (figure 2). The latter indicating that HBOT for 10 consecutive days already generates a change in the proliferation dynamics of the ISCs within the crypts.

Using the specific ISCs marker, Olfm4, we observed that stem cells co-distribute with the proliferation marker BrdU. Of note, another cell population shows proliferative labeling but no Olfm4 staining (figure 1E). Thus, we cannot discard the possibility that HBOT can stimulate

proliferation of progenitor cells and/or emergency ISCs. The recruitment of these cells together with ISCs would represent an interesting broader effect of HBOT. Keeping in mind that HBOT has recently reported to increase the proliferation of progenitor cells *in vitro* (Benincasa et al; 2019), our result would be the first in describing similar results *in vivo* in the intestinal niche. On the other hand, if, in addition, the target of acute HBOT are the so-called emergency ISCs, a population that has been described as quiescent (Muñoz et al; 2012), this could mean that the treatment can promote their recruitment to ISCs. The possible relationship between the quiescent and the actively cycling ISCs and the biological consequences of their HBOT response needs to be further explored.

The increase in proliferation in H20D occurred in a synchronous fashion (figure 1K). This means that along the duodenum all the H20D crypts presented a similar and higher number of BrdU+ cells than the control or H10D groups. This synchronicity could emerge as a consequence of cell cycle arrest provoked by HBOT or by the maximization of the proliferative capacity of the crypt's cells. As shown by previous authors, identification of the BrdU and PHH3 proportion allows for evaluation of cell cycle arrest (Cappella et al; 2008 and Ren et al; 2018). Based on these works we analyzed the proportion of the aforementioned markers in the C20D and H20D groups and determined that HBOT does not arrest the cells in a particular stage of the cell cycle. Thus, HBOT maximizes the proliferation mode in the intestinal crypts.

It is critical to note that these effects were not generated by an increase in ATP production due to an enhanced oxidative metabolism by an increment in available oxygen, as revealed by the analysis of the enzymatic activity of COX and CS (figure 3 B-C). These results were in concordance with our previous work, where we showed that using HBOT for 10 consecutive days did not change the basal metabolic rates of mice (Peña-Villalobos et al; 2018). They are also in line with a recent *in vitro* analysis of lung fibroblast where no changes in CS activity were reported (Dejmek et al; 2018). Notwithstanding, it is remarkable how a treatment that increases oxygen tension does not alter oxidative metabolism in the intestinal epithelium while other reports indicate that acute HBOT does

generate changes in tissues like brain or skeletal muscle (Matchett et al; 2009 and Ishihara; 2019). If HBOT can generate a differential physiological response in tissues and organs warrants further investigation.

mTORC1 is known to sense oxygen levels (Lane et al; 2017) and to regulate stem cell proliferation (Murakami et al; 2004 and Hu et al; 2017). The stimulation of S6K1 by mTORC1 has been widely reported. In addition, mTORC1 has been characterized as a rapamycin sensitive complex. Strikingly, we found that HBOT can recover rapamycin induced mTOR pathway inhibition, as shown in figure 4A and supplementary figure 1. Furthermore, analysis of BrdU+ cells per crypt showed that HBOT and rapamycin increased proliferation of the cells within the crypt independently. We attribute this effect of rapamycin to its capacity to mimic a CR. Under CR, Paneth cells, forming part of the stem cell niche, sense a downregulation of mTORC1 and generate a paracrine signal (NAD synthesis) stimulating ISCs to proliferate (Igarashi & Guarente; 2016 and Peña-Villalobos et al; 2019). HBOT also increases proliferation through mTORC1, but, at this point, we would like to suggest that it probably occurs through a different and competitive way of rapamycin's action. When both treatments are combined, there is no increase or synergic effect between them. On the contrary, they cancel each other's effects (figure 4), resulting in no differences in the number of BrdU+ cells per crypt and the frequency distribution of ISCs (figure 5A, 6F). Therefore, HBOT can modulate the proliferation of ISCs (and most likely progenitor cells) in the crypt acting through mTOR pathway signaling. The possible intricate network of the interplay between these two metabolic mTORC1 stimuli with similar output warrants further investigation.

The intrinsic metabolism of adult stem cells that reside in a hypoxic condition like neural stem cells in the subventricular zone or mesenchymal stromal cells in the bone marrow seems to be maintained by an aerobic glycolysis but without generating high levels of reactive oxygen species (ROS) and therefore maintaining a low proliferation rate that preserves the self-renewal potential (Shyh-Chang & Ng; 2017). But when these cells sense a high ROS environment, they undergo an active

proliferation and differentiation process (Tothova et al; 2007). In *Drosophila*, ISCs also undergo hyperproliferation in response to high ROS levels (Hochmuth et al; 2011). This high ROS level can be produced by an increase in mitochondrial activity which in turn can be activated by an increase in mTOR signaling. On the other hand, it has been proposed that changes in the REDOX state of stem cells, induced by changes in the electron transport chain due oxygen reduction, can change their proliferative behavior and might have effects at nuclear epigenetic and gene expression (Wei et al; 2018). Therefore, the increase in ISCs proliferation in mice could be explained by a response to an increase in ROS levels or changes in REDOX state. Our group is currently investigating the relationship between HIF1-α, ROS levels and mTORC1 as a candidate mechanism to explain the effects in response to hyperbaric oxygen.

The metabolic program of stem cells is intrinsically determined and essential for the maintenance of those cells, impacting the balance of self- renewal, and differentiation. HBOT has positive effects in bone marrow stem cells migration and mesenchymal stem cells proliferation (Thom et al; 2006 and Geng et al; 2015). Here we show that HBOT can be also used to increase the proliferation of ISCs and progenitor cells through mTORc1 pathway signaling, without altering the oxidative metabolism of the animal's small intestine. Results in line with recent findings pointing to the capacity of mTORc1 to regulate the proliferation state of post-natal skeletal stem cells (Newton et al; 2019). Most likely, since HBOT promotes a systemic effect and other tissue-resident stem cell pools are affected. Future studies need to address a possible HBOT/mTORC1 signaling on other somatic stem cells populations.

In summary, our findings indicate that HBOT on its own can mimic the effects of rapamycin on proliferation of ISCs. Noteworthy, HBOT has the advantage of being a non-invasive and relatively cheap treatment, favoring its use in future therapies to regenerate damaged intestinal epithelia.

## Conclusions

HBOT for 20 consecutive days potentiates ISC proliferative behavior, such increase in proliferation occurs in a synchronous fashion all along the duodenum without altering oxidative metabolism, suggesting that HBOT maximizes the proliferation rate of ISC. Our results show that mTORC1 pathway modulation is responsible for this HBOT driven increase in proliferation. When mTORC1 is inhibited by rapamycin, ISCs undergo a caloric restriction like situation, that in mice generates similar effect to that observed if they had been treated with HBOT for 20 consecutive days. However, when both treatments were combined, no effect on ISC proliferation were detected, suggesting that HBOT effect were modulates through mTORC1 pathway, and due its inhibition, the effect disappears. These results shed light on the molecular mechanisms underlying HBOT therapeutic action, a medical treatment that has been used for a long time but whose subjacent mechanisms remain unclear.

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Author contributions

**Conceptualization:** ICM, VP & IPV

**Investigation:** ICM, IPV & PL

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**Writing-Original Draft:** ICM; IPV & DA

**Writing-Review & Editing:** VP & PL

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# Figures

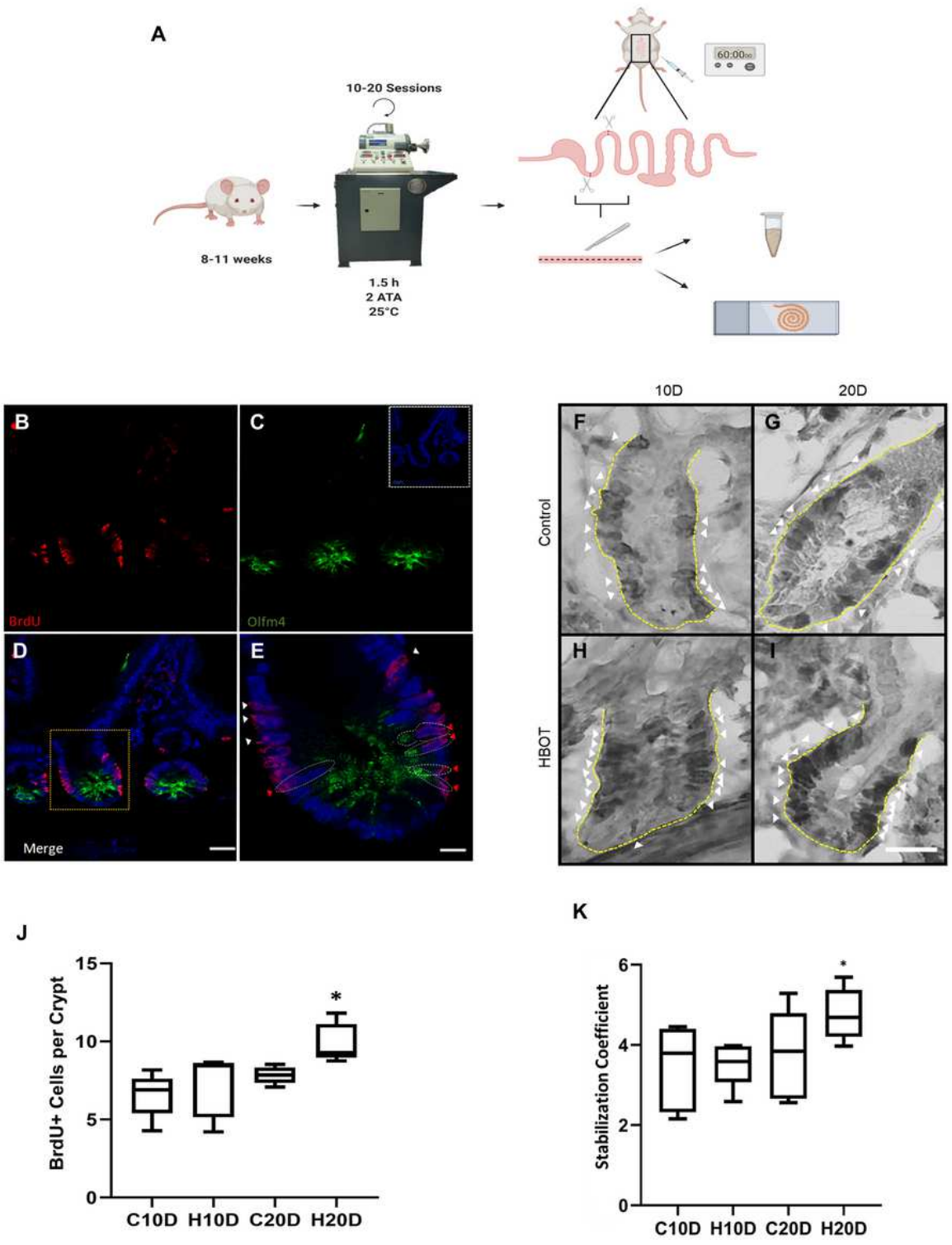



**Figure 1**

HBOT increases ISCs proliferation in a synchronous fashion. A) Schematic workflow of HBOT treatment and experimental procedures on small intestine samples. B-E) Representative immunofluorescence images of ISC proliferation in intestinal crypts. B) Proliferative cells were detected by BrdU labeling (Red).

C) ISCs labeled as Olfm4+ cells, a cytoplasmatic marker (Green). Inset shows DAPI nuclear staining (Blue). D) Merged image. Yellow box indicates crypt with proliferating ISCs among other cell populations. Bar= 15 gm. E) Zoom of yellow box from image D. Red arrowheads indicate proliferating ISCs while white arrowheads indicate other proliferating cells within the intestinal crypt. Bar= 30 gm. F-G) Representative immunohistochemistry images of intestinal crypts with proliferating BrdU+ cells; experimental groups as indicated. Nuclei labeled by hematoxylin counterstaining. Bar= 20 gm. Yellow dotted lines outline the crypts while white arrowheads show BrdU+ cells. 1) Quantification of BrdU+ cells per crypt for each treatment. Asterisk indicate statistical significance. Permutation test. pc 0.05. n=5. K) Stabilization coefficient indicating the level of variation inside each group. Asterisk indicates statistical significance. Only H2OD shows significant differences, indicating synchronicity. Permutation test. p<0.05. n= 4.

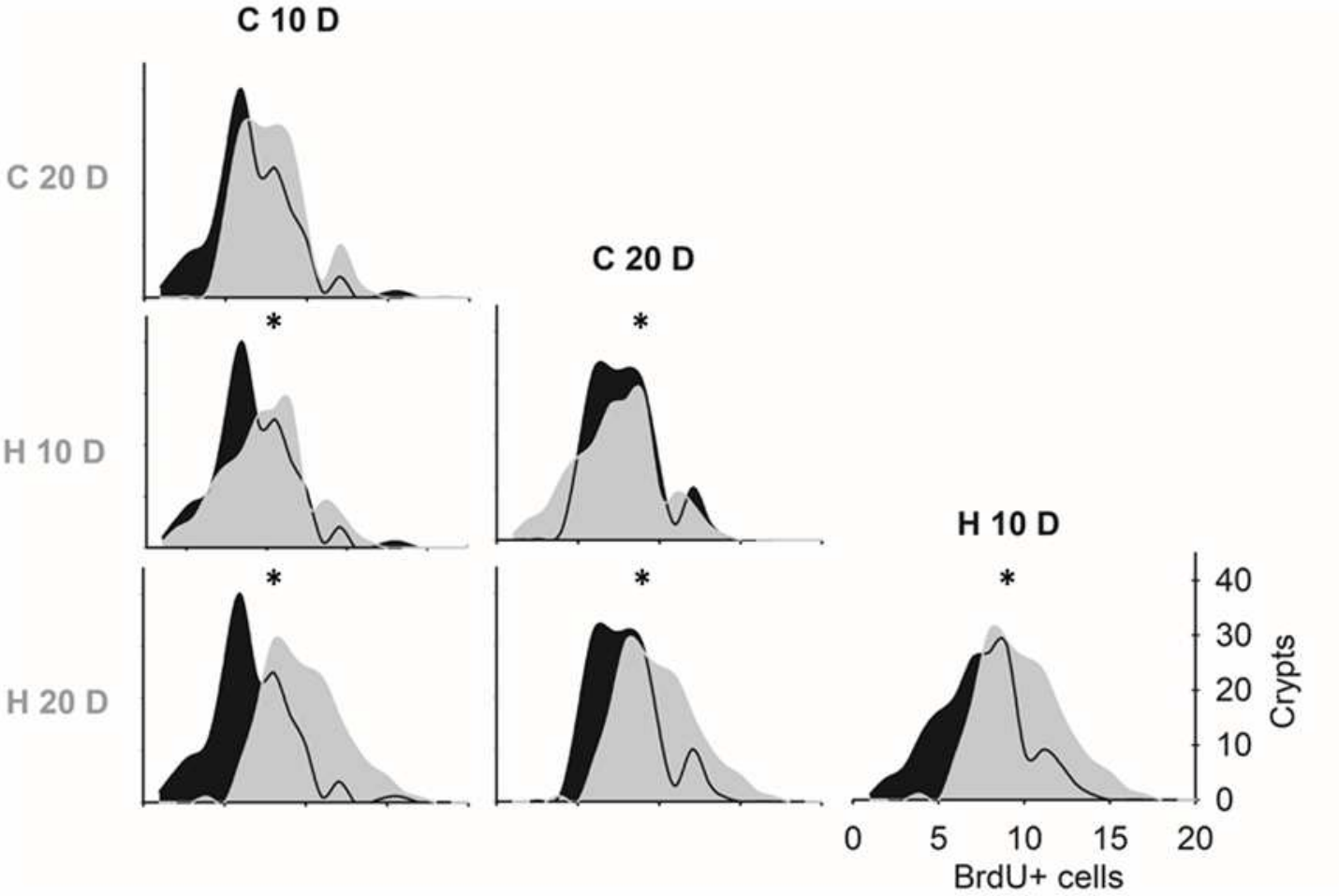
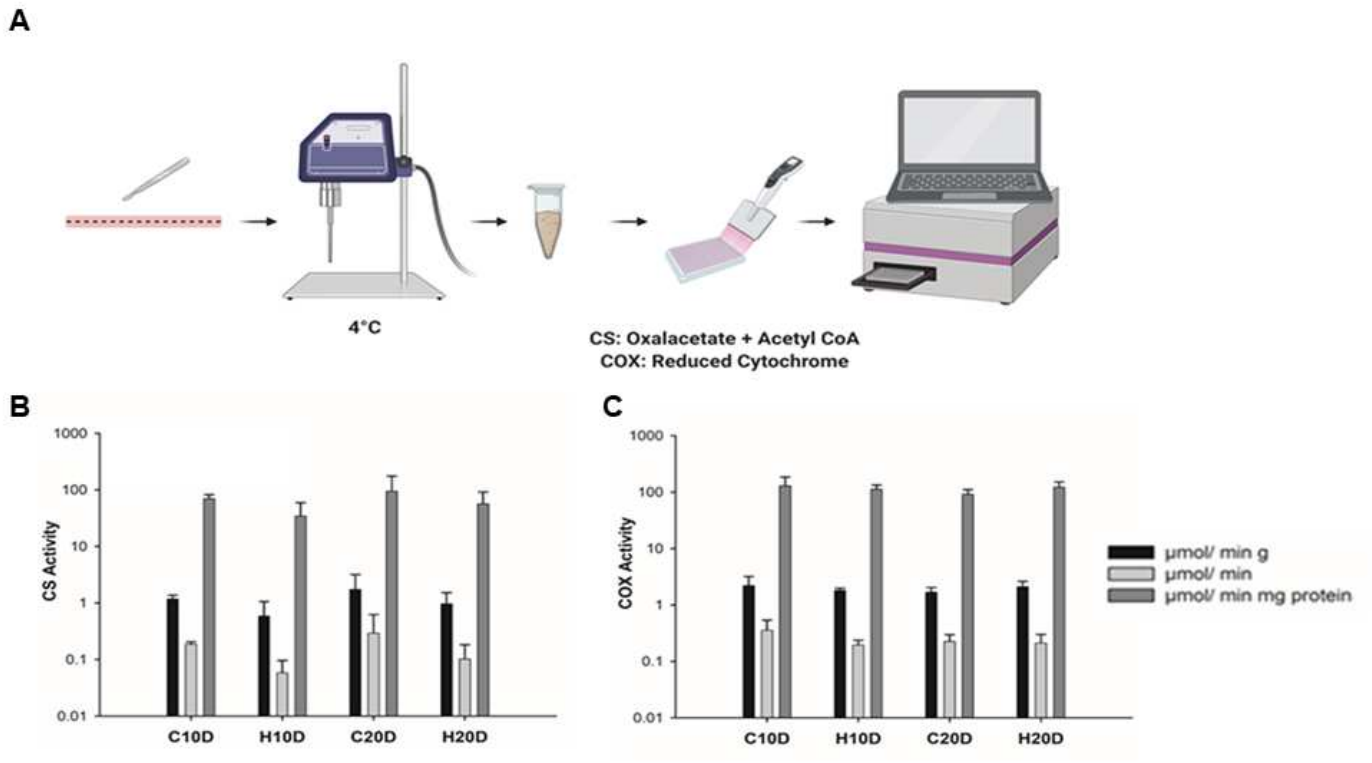


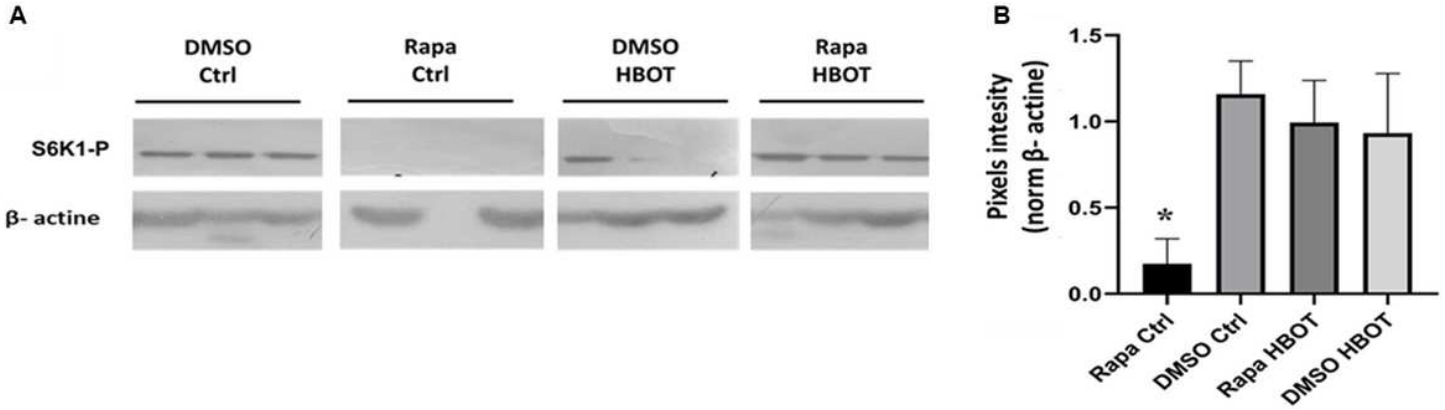

**Figure 2**

HBOT has an accumulative effect on time. Comparison of frequency distribution of Bra], cells per crypt between groups as indicated. Asterisk indicates differences (p < 0,05). Kohnogorov-Smimov test. Only the comparison between control groups shows no differences. n=4.




## Figure 3

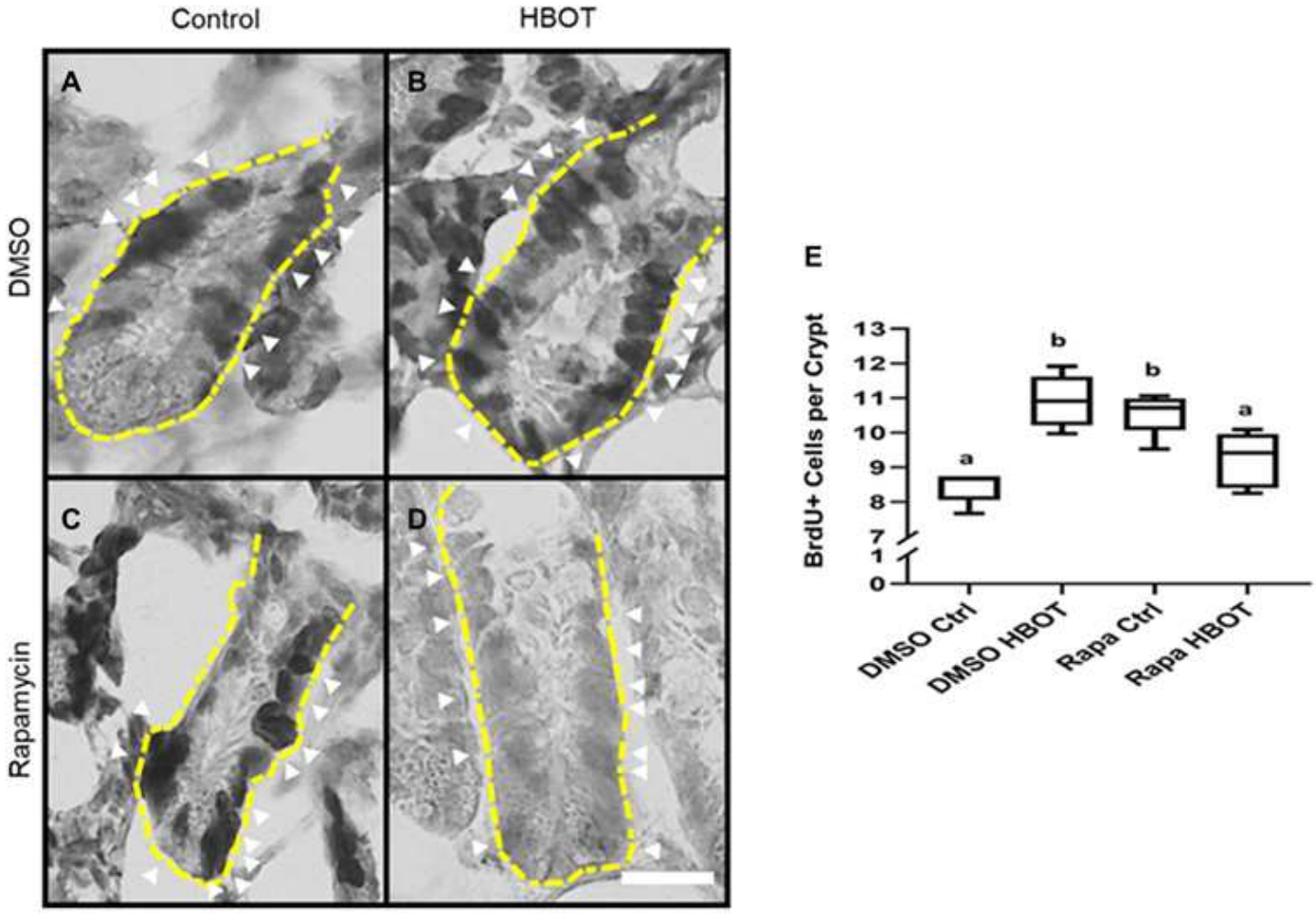
Aga. SNOT does not affect enzyme. activity In the small Intestine. A) Woncilow of enzymatic assay Cr CS sod COX activity in small inhffine tissue. 13) Bar graph of CS activity finnoknin) under each trealment. No significant differences were found. (p> 0.05). nw 3. C) COX activity (pmoUmin) under each treatment. No significant differences were found. Black bars indicate activity per gram of tissue, dark grey bars indicate activity per total proteins in the sample and tight grey bars indicate total activity. (pe 0.135). n= 3.




## Figure 4

HBO7 renovers rapamycin induced mTOR pathway inhibition. A) Western blot for phosphorylated S6101 (SliK1.14) and 13-actine in each experimental group (triplicate). First row shows DMSO control group. Second row shows rapamycin control group. Third row shows rapamycin and HOOT group. Last row

shows DMSO and MOT group. SPOT sessions for 20 days. B) Quantification of pixel Wensity for SCr141-P presence in each group normalized with internal control p.n.s. Asterisks indicates statistical significance. Permutation hst.p<0.05, r, 2 per grow.




## Figure 5

HBOT and rapamyelo increase ISCs proliferation independently. A-D) Representative immunobistochemistly images for proliferating cells. label. by Brdu incorporation. in intestinal crypts for each experimental group. 20 days of treatment in each group. Nuclei labeled by heinatoxylin cramterstaining. Bar— 20 inn. Yellow dotted lines outline the crypt periphay. White arrowhinids show Brdfft E) Quantification of Brdlrt cells for each treatment Same letters indicate no statinical significance while different letters indicate statistical significance. PermuNtion test. p<0.05. 11-5 per group.

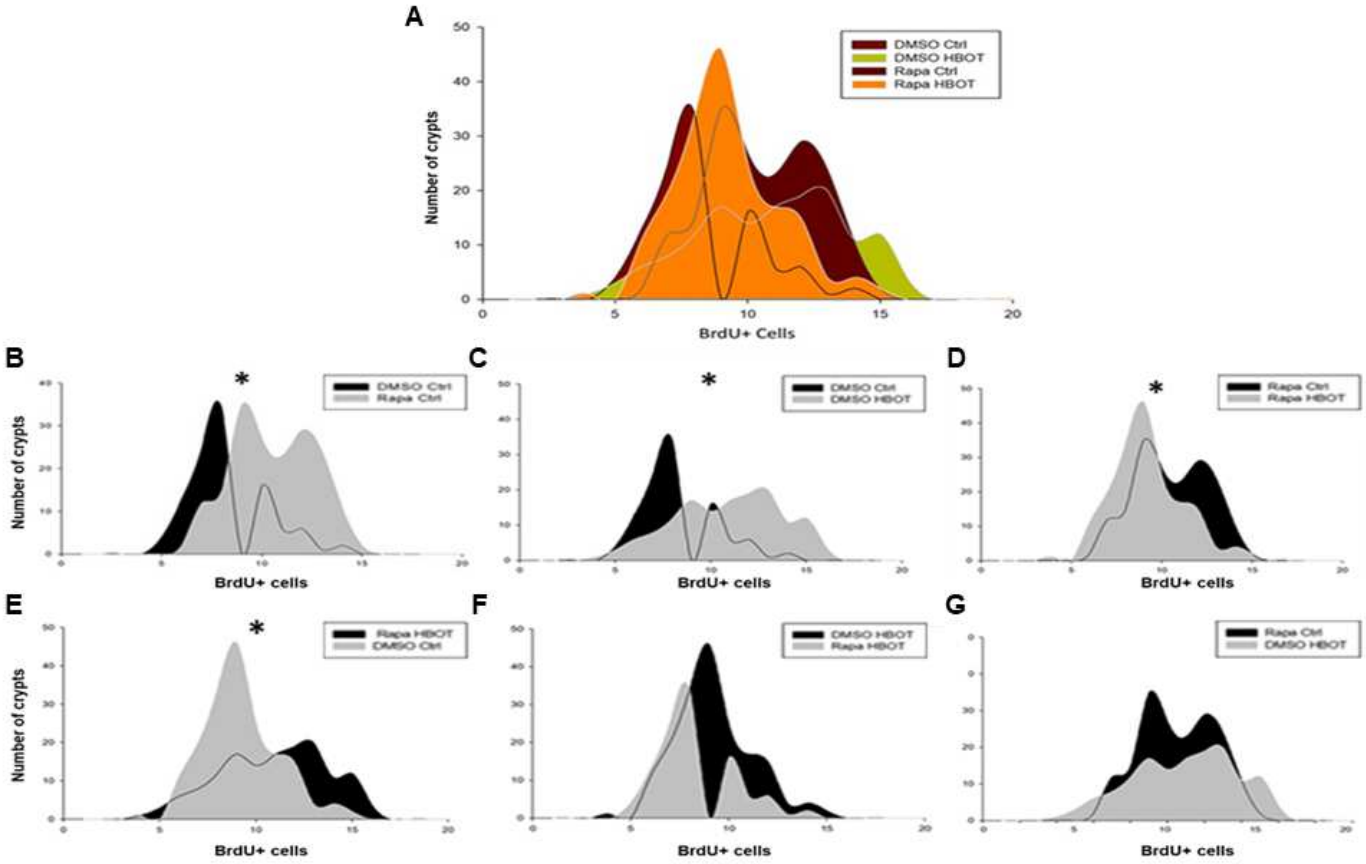



**Figure 6**

HBDE can simulate a caloric restriction. A) Superposition of frequency distribution for each Reap. All comparison was made by a KolmogorovsSmirnov test. B) Comparison between DMSO CPI and Rape Cid. pm 0.004 C) Comparison between DMSO CU) and DMSO 1180T. m 0.003 D) Comparison between Rape CPI and Raper BOOT. m 0.013 E) Comparison between DMSO Cirl and Rape BOOT. 0.009 r) Comparison between Rape 11BOT and DMSO Ctd. p>0.05 G) Comparison between Raper Crd end DMSO 11BOT. p>0.05. n= 5.

## Supplementary Files

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