The effect of hibernating phase serum on osteoclastogenic and osteogenic differentiation *in-vitro* in Japanese black bears

（ツキノワグマにおける *in-vitro* での破骨細胞および骨芽細胞への分化に対する冬眠期血清の効果）

by

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Abbreviation

ABSM: Active bear serum containing medium

ADSCs: Adipose derived stem cells

ALP: Alkaline phosphatase

ANOVA: Analysis of variance

BMM: Bone marrow derived monocytes/macrophages

BMSCs: Bone marrow stem cells

BSA: Bovine serum albumin

BSALP: Bone specific alkaline phosphatase

HBSM: Hibernating bear serum containing medium

M-CSF: Macrophage colony stimulating factor

OCs: Osteoclasts

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PTH: Parathyroid hormone

RANKL: Receptor activator of NF-kB ligand

SE: Standard error

TRAP: Tartrate resistant acid phosphatase

Note:

- The content for chapter 1 of the thesis has been published in PLoS ONE:

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Preface

My interest in hibernation:

Hibernation is a very impressive natural phenomenon. My interest in hibernation was first sparked when I was a teen reading an old story about a few men who together with a dog stayed in a cave, and went through a deep sleep for many years, after which they revived. This story made me curious about sleep, or better to say "long-term sleep". Years after that, when I enrolled the veterinary school in Iran, I became acquainted with hibernation and circadian rhythms. To complete my thesis for veterinary course in Iran, I participated in a project about bone regeneration. I did not find the opportunity to work on hibernation until I entered the Lab of Wildlife Biology and Medicine, at Hokkaido University to do my PhD studies. This time, I could work on a project on hibernation and bone biology. I developed methods to collect samples from captive bears, and to make laboratory analysis. Fortunately, I managed to conduct a project on hibernating bears in which bear cells were cultured with active and hibernating bears' serum. This research will be of the interest to biologists in various disciplines such as wildlife biology, bone biology, regenerative medicine, and physiology.

I am happy that one of my dreams about studying on "long-term sleep" came true. Also, I am glad that I contributed to a project that established sample collection and culture methods for a native bear species, the Japanese black bear (*Ursus thibetanus japonicus*), which should be further investigated.

Hibernation and inactivity:

Hibernation is a long-term torpor associated with a tremendous reduction in metabolism to save energy. Hibernation is governed by external cues such as low ambient temperature, food paucity, and day length, and internal cues such as biological clock. Heart rate, respiration and body temperature markedly decrease in hibernating mammals. Hibernating bears, however, maintain their body temperature close to normal rate. In addition, hibernating bears have other distinct features in comparison with other hibernators, for instance, they do not intake food, urinate, defecate, and stay inactive for 4 to 5 months (Tøien et al., 2011; Tsubota et al., 2008b). Prior to hibernation, bears intake large amount of food to provide energy storage for a long-term fasting in winter. Later on, bears go through a transitional period in which they reduce their foraging behaviour and physical activity, and prepare their den/hibernaculum for winter torpor. Majority of dens are ground dens, burrowed in hillsides or under trees or bushes (Beecham et al., 1983), which are located in bears summer home range (Kolenosky and Strathearn, 1987). Interestingly, bears have about 5 months delayed implantation, favouring the mother to become pregnant (lasting 2 months), and to give birth within hibernation period; the time when mothers feed and

nurse their cubs while male bears are usually hibernating and do not intrude (Rogers, 1977; LeCount, 1983). Upon the end of hibernation, bears go through another transitional period to resume foraging, and replenish energy. Such a life cycle is unique among mammals. Bears undergo a long-term torpor while do not suffer from health issues such as metabolic or musculoskeletal disorders.

In general, bears' prolonged inactive phase begins prior to hibernation when bears become lethargic and reduce their diurnal activity, followed by denning (hibernation) period when bears barely move, and proceeds to the following days of emerging from the den when bears are not fully active yet (Lindzey and Meslow, 1976; Beecham et al., 1983). In total, physical activity ratio in these periods is one sixth of non-denning phase (Lindzey and Meslow, 1976). The inactivity is primarily triggered by low ambient temperature and increased precipitation (Lindzey and Meslow, 1976; Kolenosky and Strathearn, 1987). Bears residing in areas with poor food access go through dormancy earlier than the bears with good food condition (Rogers, 1977). Moreover, it has been reported that in areas with sufficient food access bears keep foraging until the first heavy snow, indicating food shortage and precipitation as important triggers for denning (Jonkel and Cowan, 1971). Females tend to initiate denning earlier than males; however, it may vary depending on severity of the winter or the location of bears' population (Lindzey and Meslow, 1976; Kolenosky and Strathearn, 1987). On the contrary, males emerge from the den earlier than females, partly attributed to the increased day length, ambient temperature (Lindzey and Meslow, 1976; Kolenosky and Strathearn, 1987), and probably testosterone level (Tsubota and Kanagawa, 1989; Tsubota et al., 1997).

Disuse osteoporosis:

Osteoporosis refers to a pathologic condition associated with poor bone mass density and an imbalance in bone turnover; bone formation and resorption. Over 200 million people worldwide are suffering from osteoporosis (Sözen et al., 2017). With the increasing trend of osteoporosis (Dhital et al., 2019), this disease can lead to severe frailty and bone fracture inflicting mortalities and extensive costs, e.g. annual $17.9 billion and £4 billion in the USA and UK, respectively (Huntjens et al., 2014; Clynes et al., 2020).

Osteoporosis is caused by various etiologies such as genetic, metabolic (e.g. hyperparathyroidism, and diabetes), postmenopausal, aging, and disuse (e.g. unloading, microgravity, and long-term inactivity) (Janghorbani et al., 2007; Kwan, 2015). Disuse osteoporosis refers to the status in which bone density is diminished (mostly) due to reduced or lack of mechanical load to the bone. Bones receive the mechanical cues mainly from weight bearing, interactions with the ground, and muscular pressures. These factors primarily exert their effects through interstitial fluid flow to osteocytes, as important mechanoreceptors in bone (Tami et al., 2002; Giannotti et al., 2013; Gatti et al., 2018). The reduction or absence of mechanical load can disturb bone turnover through different mechanisms, e.g. i) Lack of mechanical cues

causes osteocyte apoptosis which elicits osteoclasts recruitment, leading to increase in bone resorption (Li et al., 2005b; Aguirre et al., 2006). ii) Unloading increases sclerostin, primarily produced by osteocytes, culminating with reduced bone formation (e.g. as antagonist to Wnt) and increased bone resorption (Shahnazari et al., 2012). iii) NF-kB1, produced by osteocytes, osteoblasts and/or muscle cells in unloading conditions, increases osteoclast activity and decreases osteogenesis (Nakamura et al., 2013). iv) Vascular degeneration due to microgravity leads to ischemia of bones (Doty et al., 1990).

Given the large number of osteoporotic patients and the growing trend in aged population, which is associated with reduced physical activity, it is highly essential to find ways to control and treat osteoporosis. Studies on disuse have demonstrated that only a short period of unloading or disuse significantly reduces bone formation and increases bone resorption, for instance i) 7-day head-down tilt bed rest in humans (Lueken et al., 1993), ii) 28-day limb unloading in ovine model (Gadomski et al., 2014), iii) 28-day limb immobilization in dogs (Waters et al., 1991), and iv) 15 to 21 days of limb unloading in mice (Judex et al., 2004), resulted in significant bone loss. Interestingly, despite long-term inactivity, hibernating mammals do not suffer from osteopenia or osteoporosis (Bogren et al., 2016; Doherty et al., 2016). That is, hibernating mammals can serve pragmatic models to resist against osteoporosis; a model that provides important insights for regenerative medicine and bone biology.

Chapter 1

Hibernating bear serum hinders osteoclastogenesis *in-vitro*

Abstract:

Background: Bears do not suffer from osteoporosis during hibernation, which is associated with long-term inactivity, lack of food intake, and cold exposure. However, the mechanisms involved in bone loss prevention have scarcely been elucidated in bears.

Materials and methods: I investigated the effect of serum from hibernating Japanese black bears (*Ursus thibetanus japonicus*) on differentiation of peripheral blood mononuclear cells (PBMCs) to osteoclasts (OCs). PBMCs collected from 3 bears were separately cultured with 10% serum of 4 active and 4 hibernating bears (each individual serum type was assessed separately by a bear PBMCs), and differentiation was induced by treatment with macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL).

Results: PBMCs that were cultured with the active bear serum containing medium (ABSM) differentiated to multi-nucleated OCs, and were positive for TRAP stain. However, cells supplemented with hibernating bear serum containing medium (HBSM) failed to form OCs, and showed significantly lower TRAP stain (p-value < 0.001). On the other hand, HBSM induced proliferation of adipose derived mesenchymal stem cells (ADSCs) similarly to ABSM (p-value > 0.05), indicating no difference on cell growth.

Conclusions: It was revealed that osteoclastogenesis of PBMCs is hindered by HBSM, implying an underlying mechanism for the suppressed bone resorption during hibernation in bears. In addition, this study for the first time showed the formation of bears' OCs *in-vitro*.

Introduction:

Hibernation is a phenomenon affected by external stimuli such as cold season, short day length and food paucity, and internal factors such as biochemical regulations and circadian rhythm. Hibernating mammals have evolved with various types of physiological and behavioral responses, which enable them to go through harsh environmental conditions. Bears, in terms of body mass, are by far the largest hibernating mammalian species. Another distinctive feature is that bears do not intake food, urinate, nor defecate during hibernation. Interestingly, hibernating bears' musculoskeletal system is not compromised by environmental and metabolic alterations throughout the torpor period (McGee–Lawrence et al., 2008; Doherty et al., 2014; Malan, 2014).

Previous studies by the use of biochemical, histological, and imaging tests have clearly shown that bears do not suffer from osteoporosis despite long-term inactivity, lack of food intake, and cold exposure during hibernation (Floyd and Nelson, 1990; Pardy et al., 2004; McGee et al., 2008; McGee–Lawrence et al., 2009; Seger et al., 2011; Fedorov et al., 2012; McGee–Lawrence et al., 2015). Similar conditions in humans and other mammals lead to osteopenia and osteoporosis, e.g. long-term inactivity and disuse such as long-term bed bound, and microgravity increase bone resorption to a rate higher than bone formation (Brighton et al., 1985; Skerry and Lanyon, 1995; Li et al., 2005a; Alexandre and Vico, 2011; Kueper et al., 2015). The imbalance in bone remodeling culminates in various degrees of bone loss.

There is growing evidence that the serum component can reflect the state of osteoporosis, and physical activity in humans (Kasapis and Thompson, 2005; Kujala et al., 2013; Beavers et al., 2014; Zhang et al., 2018; Pontes et al., 2019). The use of osteoporotic patients' serum in osteogenic cell culture can induce osteoporotic-like conditions *in-vitro*, such as increase in the expression of receptor activator of nuclear factor kappa-β ligand (RANKL) (Li et al., 2015), a potent inducer of osteoclast (OC) differentiation, and decrease in the expression of osteogenic genes (Zhao et al., 2019).

The use of bear serum for cell culture has been investigated in recent years. The *in-vitro* experiments have indicated that the use of active and hibernating bear serum for cell culture can induce active-like and hibernation-like cell responses, respectively (Jansen et al., 2016; Rigano et al., 2017; Chanon et al., 2018). For example, the use of hibernating bear serum inhibits proteolysis, and increases protein content in muscle cells (Chanon et al., 2018).

As mentioned above, it is almost unequivocal that bears do not develop osteoporosis due to hibernation. However, the mechanisms and features that enable bears to stay safe from bone loss have largely remained unknown. Here, I present *in-vitro* evidence that serum of hibernating bears suppresses osteoclastogenesis of bear peripheral blood mononuclear cells (PBMCs). PBMCs, akin to bone marrow monocytes, are considered as progenitor cells for osteoclasts. Also, here for the first time I report the formation of bear OCs *in-vitro*.

Materials and Methods:

Sampling from animals

Samples were collected from captive adult Japanese black bears (*Ursus thibetanus japonicus*) at Ani Mataginosato Bear Park (Kuma Kuma En) located in Akita Prefecture, Japan (N 39.915398 E140.536294), in active and hibernation periods. Bear information and sample types are presented in Table 1–1. The bears are routinely fed by crushed corn, acorn, chestnut, and a kind of local Japanese butterbur. During hibernation period, the bears do not have access to food, while they have access to water *ad libitum*. Bears hibernation was characterized by lack of movement and food intake. Hibernation in these captive bears reportedly begins in December and proceeds to around early April, when bears resume activity and foraging (Tsubota et al., 2008a,b). All procedures and animal care were conducted in accordance with the Guideline of the Animal Care and Use of Hokkaido University and were approved by the Animal Care and Use Committee of Hokkaido University (Permit Number: 18-0179). To collect samples, bears were anesthetized with 40 µg/kg medetomidine hydrochloride (Domitor; Zenoaq, Japan) and 3.0 mg/kg zolazepam hydrochloride and tiletamine hydrochloride cocktail (Zoletil; Virbac, France) intra-muscularly. Blood was collected from the jugular vein into plain tubes, and EDTA containing tubes for serum collection and peripheral blood mononuclear cells (PBMCs) isolation, respectively. Serum was isolated from plain tubes and preserved at -80°C. EDTA containing tubes were delivered at 4°C to the lab for PBMCs isolation, as explained below. Adipose tissue was biopsied from the subcutaneous inguinal and sternal areas, put in Dulbecco's Modified Eagle's Medium (DMEM, FUJIFILM Wako Pure Chemical Corporation, Japan), and delivered at room temperature to the lab for adipose derived mesenchymal stems cells (ADSCs) isolation, as explained below.

Table 1–1.
Bear information, sample type, and collection season.

Bears ID	A	B	C	K	D	E	F	G	C	D	H	I
Type of sample	PBMCs	PBMCs	PBMCs	ADSCs	Serum	Serum	Serum	Serum	Serum	Serum	Serum	Serum
Season	May	May	July	July	May	May	May	May	January	January	January	January
Age (year)	7	7	19	23	18	12	26	16	19	18	18	17
Body mass* (kg)	86	90.5	143	157	145.5	153	107	78	157.5	166	135.5	201

All samples were collected from male bears, except bear G, a female bear. May and July samples are for active phase. January samples are for hibernation phase. The average of active and hibernating bears' age (for serum samples) is 18.0 ± 2.9 and 18.0 ± 0.4, respectively. The difference between the groups' age is not significant; *p*-value = 1.00. Data were assessed by two-tailed Student's *t*-test.

PBMCs isolation

The blood samples were obtained from 3 adult male bears A, B, and C in active season (May- July). In the lab, the blood (collected in EDTA containing tubes) collected from each bear was separately diluted with phosphate-buffered saline (PBS) to the ratio 1:1. Blood-PBS mixture was slowly added onto Ficoll-Paque PLUS (GE Healthcare Life Sciences, Sweden) in falcon tubes based on the instruction of the product. The tubes were centrifuged at 400 × g, for 40 minutes at 20°C. The buffy coat was collected and transferred to new falcon tubes, and were centrifuged twice with PBS at 400 × g for 15 and 10 minutes at 20°C. Finally, the PBMCs from each bear were separately collected in Eppendorf tubes, and preserved in CELLBANKER 2 (AMS Biotechnology Ltd., UK) at -80°C.

ADSCs isolation

Adipose tissue was obtained from a mature male black bear (bear K) in active season. The tissues were digested by 2 mg/ml collagenase (FUJIFILM Wako Pure Chemical Corporation) in DMEM containing 2% fatty acid-free bovine serum albumin (BSA) (FUJIFILM Wako Pure Chemical Corporation) at 37 ºC for 2 hours with shaking at 90 cycles/minute. The suspension was filtered through 200-μm nylon filter and centrifuged at 200 × g for 5 minutes at room temperature. The pellet was filtered through 25-μm nylon filter, and then centrifuged at 200 × g for 5 minutes at room temperature. The pellet was collected as ADSCs, and preserved in CELLBANKER 2 (AMS Biotechnology Ltd.) at -80°C. This method is based on previously established protocol for ADSCs isolation from adipose tissue (Okamatsu–Ogura et al., 2018).

Cell culture

PBMCs were cultured at a concentration of 3 to 5 × 10^6 cells per 35 mm collagen-coated dish, in a-MEM (Minimum Essential Medium Eagle, Sigma-Aldrich, UK) supplemented with10% bear serum, 100 units/ml penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation), 50 μg/ml gentamicin (FUJIFILM Wako Pure Chemical Corporation), 2.5 μg/ml amphotericin B (FUJIFILM Wako Pure Chemical Corporation) and 2 mM L-Glutamine (Nacalai Tesque Inc., Japan), and incubated at 37°C with 5% CO_2. Protocol for the OC differentiation is summarized in Fig 1–1. The day after seeding (day 2), the supernatant was discarded, and cells either i) at a concentration of 0.2 to 0.5 × 10^5 cells per well were seeded on 96-well plate or ii) at the same dish remained to the end of experiment, were cultured with the same medium/serum type with addition of 20 ng/ml macrophage colony stimulating factor (M-CSF, Gibco, USA) for three days. On day 5 and 8, the medium was changed to new medium containing 20 ng/ml M-CSF and 50 ng/ml RANKL (Sigma-Aldrich, USA). On day 11, cells were finally washed with PBS, fixed with 10% phosphate-buffered formalin, washed with distilled water, and stained with TRAP Stain (TRAP staining kit, Cosmobio Co., LTD., Japan) to examine the presence of tartrate-resistant acid phosphatase (TRAP) by a light microscope. The stained area in each dish was quantified by the use of NIH Image J Software.

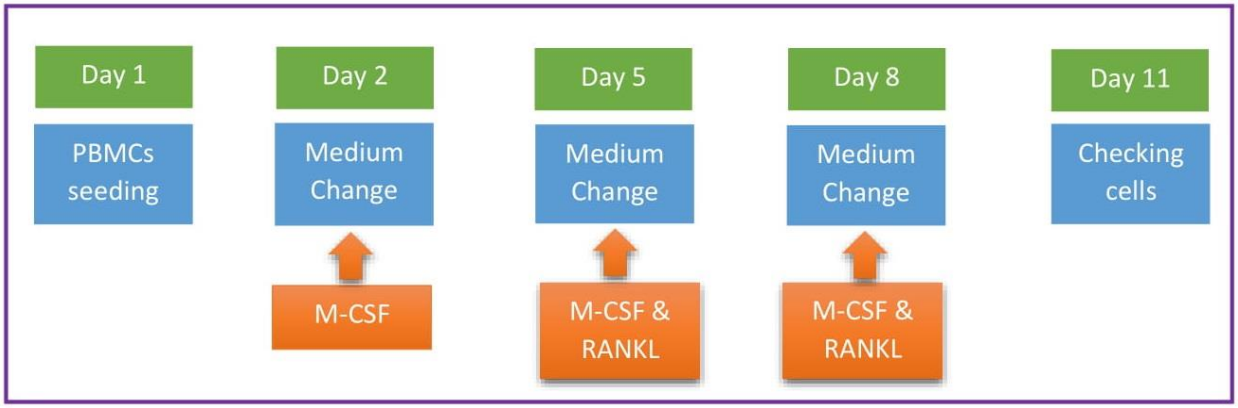

Fig 1–1. The procedure of PBMCs culture from day 1 to 11 to form OCs.

PBMCs of bears A, B, and C were separately cultured with active bear serum containing medium (ABSM) prepared separately from serum of bears D, E, F, and G, and hibernating bear serum containing medium (HBSM) prepared separately from serum of bears C, D, H, and I (Table 1–1). PBMCs of bear A and B were twice evaluated for the mentioned procedure on dish and plate. All groups were treated with MCSF and RANKL equally and similarly.

ADSCs from one bear (bear K) were cultured on 35 mm collagen-coated dish (cell concentration shown in the results) with 10% ABSM or 10% HBSM, each from 4 different individuals, as mentioned for PBMCs. ADSCs were twice cultured; after 6 days (medium changed at day 3), cells were detached by trypsin/EDTA solution, counted on hemocytometer, re-cultured (subculture), and after 4 days, detached and recounted. At each passage, ADSCs were cultured separately with each of four types of ABSM and HBMS.

Statistical analysis

Values are expressed as mean ± standard error (SE). The data were analyzed by the use of analysis of two-tailed Student's *t*-test or Analysis of Variance (ANOVA) together with a post-hoc Tukey HSD (by the use of IBM SPSS Statistics, Version 23), and significant *p*-value was considered equal or less than 0.05.

Results:

PBMCs culture and TRAP staining

PBMCs from bears A, B, and C (Table 1–1) were cultured with either ABSM or HBSM, and induced differentiation to OCs by treatment with M-CSF and RANKL (Fig 1–1). On day11, PBMCs cultured with ABSM showed multi-nucleated morphology, a typical feature of OCs. These multi-nucleated cells were positively stained for TRAP activity, indicating differentiation to active OCs (Fig 1–2 A, B). By contrast, PBMCs cultured with HBSM failed to differentiate to multi-nucleated cells (Fig 1–2 C, D). TRAP-stained area in ABSM group was 21.28 ± 3.89% of total area, being significantly (p-value < 0.001) higher than that in HBSM group (1.73 ± 0.43%) (Fig 1–3).

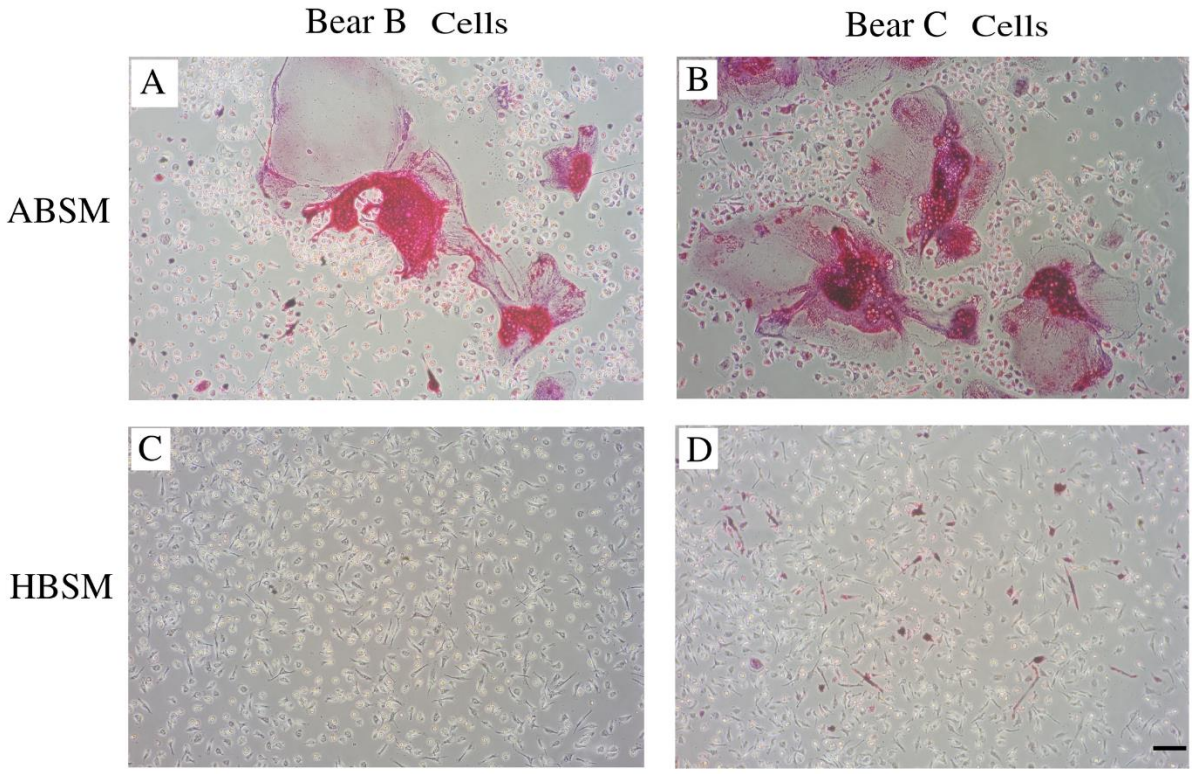

Fig 1–2. TRAP stained samples at day 11.

(A-B) cultured cells of bears B and C with active bear serum containing medium (ABSM); multi-nucleated giant osteoclasts which are TRAP stain positive. (C-D) cultured cells of bears B and C with hibernating bear serum containing medium (HBSM); non-differentiated cells, which are poorly stained with TRAP stain. Scale bar is equal to 500 μm.

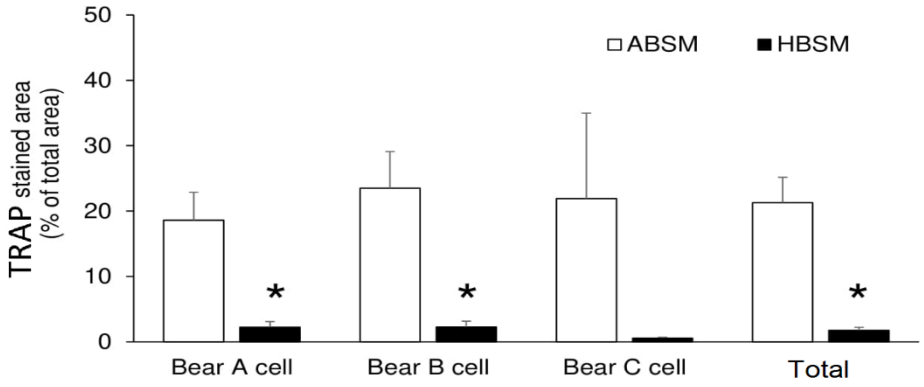

Fig 1–3. The percentage of TRAP stained areas in osteoclast cultures.

Bear A cultured with active bear serum containing medium (ABSM) (n = 4), and hibernating bear serum containing medium (HBSM) (n = 4). * *p*-value = 0.040. Bear B cultured with ABSM (n = 4), and HBSM (n = 4). * *p*-value = 0.028. Bear C cultured with ABSM (n = 3), and HBSM (n = 3). *p*-value, = 0.242. Total of three bears: ABSM (n = 11), and HBSM (n = 11). * *p*-value = 0.0005. Data were assessed by two-tailed Student's *t*-test. All values represent means ± SE.

ADSCs culture and cell number count

ADSCs were cultured with either ABSM or HBSM and effect on cell proliferation was examined. At the first passage, 6-day culture showed significant (p-value < 0.05) increase in cell number (Fig 1–4 A) and the growth rate showed no significant difference between ADSCs cultured with ABSM (2.7-fold) and HBSM (2.9-fold) (Fig 1–4 A). At the second passage, cell number significantly (p-value < 0.05) increased in both ABSM and HBSM (Fig 1–4 B). Growth rates of ABSM and HBSM groups were similar in 4-day culture at the second passage (Fig 1–4 B). ADSCs in both groups turned to spindle-shaped cells at day 6 (first culture), and day 4 (subculture/ second culture) (Fig 1–5).

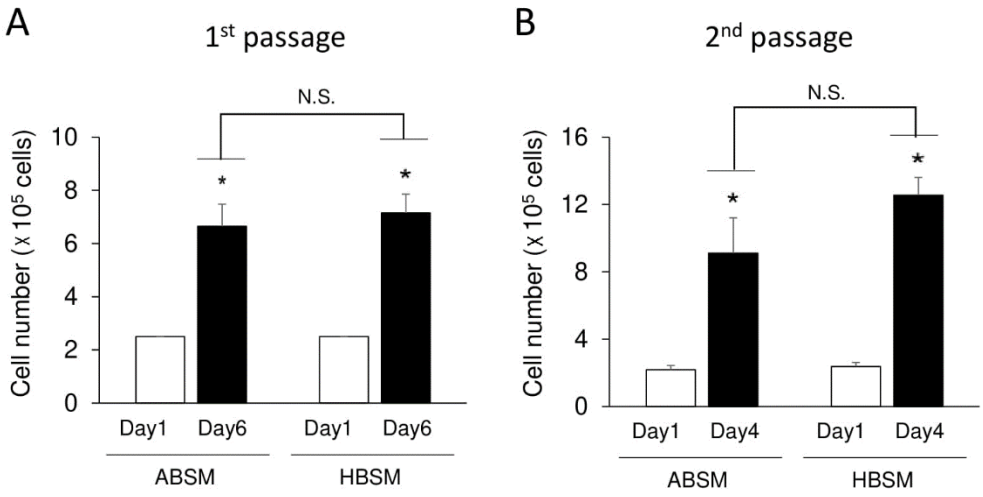

Fig 1–4. The cell number of adipose derived stem cells (ADSCs) of bear K cultured with active bear serum (separately with each serum of bear D, E, F, and G) containing medium (ABSM) and hibernating bear serum (separately with each serum of bear C, D, H, I) containing medium (HBSM) for the first and second passage.

(A) First passage; there was a significant difference between groups as determined by one-way ANOVA ($F(3, 12) = 21.80$, p-value < 0.001). Post-hoc comparisons using Tukey HSD test indicated that there was a significant difference between day 1 and 6 in ABSM group p-value = 0.001 and in HBSM group * p-value < 0.001. The difference between ABSM and HBSM at day 6 was not significant (N.S); p-value = 0.914. (B) Second passage; there was a significant difference between groups as determined by one-way ANOVA ($F(3, 12) = 18.59$, p-value < 0.001). Post-hoc comparisons using Tukey HSD test indicated that there was a significant difference between day 1 and 4 in ABSM group * p-value = 0.007, and in HBSM group * p-value < 0.001. The difference between ABSM and HBSM at day 4 was not significant (N.S); p-value = 0.224. Each column shows the average of total cell numbers of 4 separate cultures based on 4 types of ABSM and 4 types of HBSM. All values represent means ± SE.

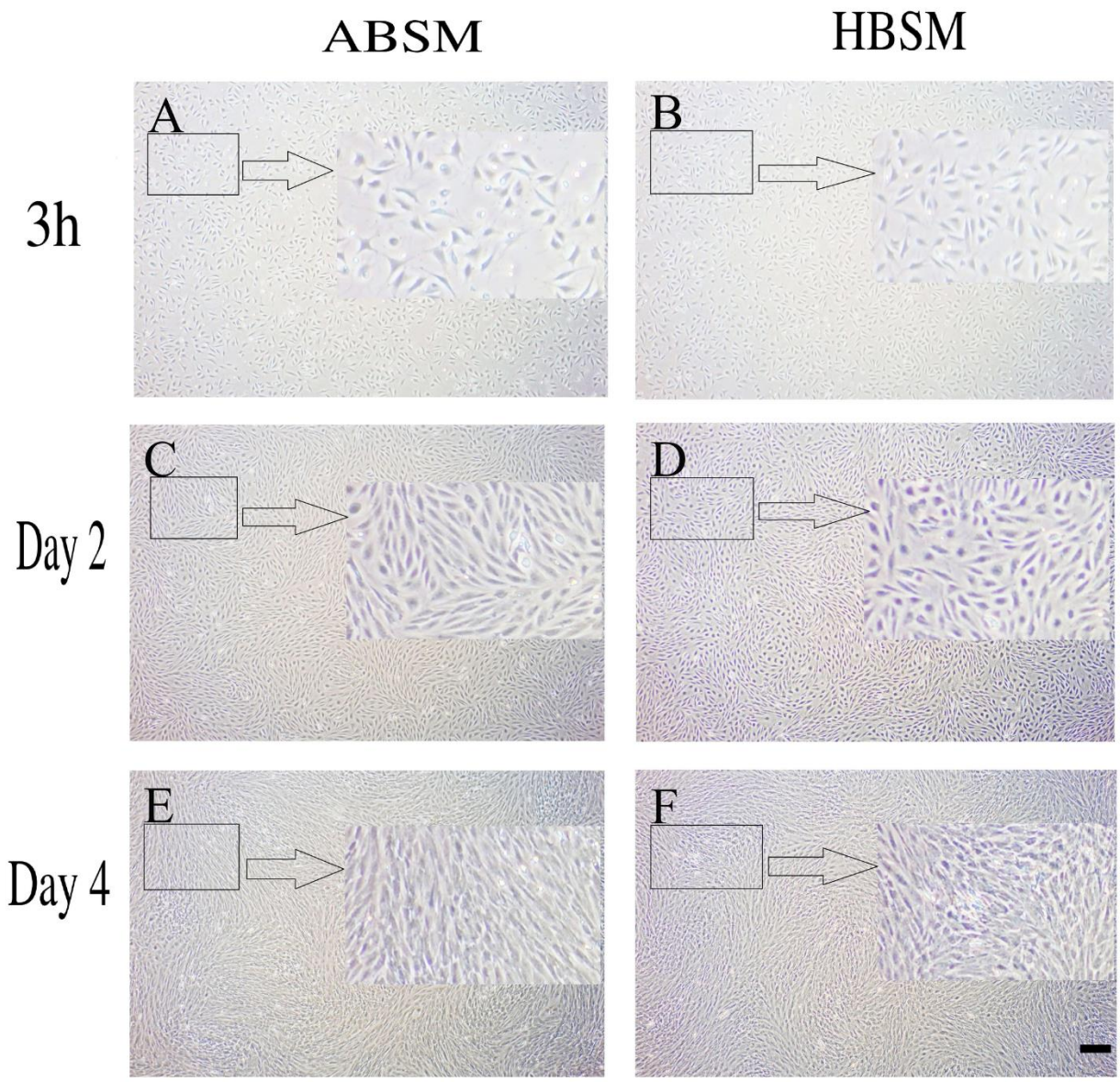

Fig 1–5. Second passage of adipose derived stem cells (ADSCs) cultured with active bear serum containing medium (ABSM) and hibernating bear serum containing medium (HBSM) in time intervals: 3 hours, day 2 and day 4 post seeding.

This indicates a similar trend in cell growth for both ABSM and HBMS. Scale bar is equal to 500 μm. Arrows show 100% zoomed areas in the square.

Discussion:

PBMCs and bone marrow derived monocytes/macrophages (BMM) are ubiquitously used for *in-vitro* osteoclastogenesis. Previous investigations have presented *in-vitro* OC formation from different mammalian species such as humans (Terkawi et al., 2018), primates (Roodman et al., 1985), rodents (Mouline et al., 2010), dogs (Wijekoon et al., 2018), cats (Ibbotson et al., 1984; Muzylak et al., 2007), rabbits (David et al., 1998), horses (Gray et al., 2000), and pigs (Herold et al., 2002). To the best of my knowledge, the current work is the first to report bears' OC formation *in-vitro*. In this study, I used bears' PBMCs for OC formation *in-vitro*. In order to obtain PBMCs, only blood should be collected, which is a low risk, less invasive and inexpensive method in comparison with bone marrow collection method for isolation of BMM, particularly for wild animals such as bears.

In *in-vitro* culture, under the influence of M-CSF and RANKL treatment, PBMCs differentiate to macrophage/pre-OCs, which is followed by cell fusion. Mature and functional OCs are characterized by the presence of multiple nuclei in a very large unified cell (multi-nucleated giant cells) which produce TRAP (TRAP stain positive) (Fig 1–2 A, B) (Grasser et al., 2003; Newa et al., 2011; Bernhardt et al., 2017; Abdallah et al., 2018; Quan et al., 2018).

Here I demonstrated that bears' PBMCs that were cultured with ABSM could differentiate to OCs and were significantly TRAP positive. However, PBMCs that were cultured with HBSM failed to differentiate to OCs, and were almost negative to TRAP staining (Fig 1–2, 1–3). This is consistent with a previous study demonstrating that denning (hibernating) bears' serum derived components (isolates) inhibited chicken BMM from OCs formation (Nelson et al., 2006). Biochemical experiments indicated that the genes which contribute to OCs formation and differentiation (such as *Ostf1*, *Rab9a*, and *c-Fos*) are underexpressed in hibernating bears (Fedorov et al., 2012). Also, the level of serum TRAP (a bone resorption biomarker) is significantly lower in hibernation period than before and after hibernation in bears (McGee–Lawrence et al., 2015). In addition, histological and imaging tests revealed that trabecular and compact bone mass of the front and hind limbs are not compromised in hibernating bears. The bone mass preservation has been suggested to be related to a reduced, yet balanced, bone turnover during hibernation (Floyd and Nelson, 1990; Pardy et al., 2004; McGee et al., 2008; Seger et al., 2011).

In the present work, I observed that HBSM group could not differentiate into OCs. Both ABSM and HBSM groups were treated similarly; the only variable was the type of serum which was either hibernation or normal type. This could, in part, made me speculate that "serum factors that modulate cell differentiation are compromised during winter dormancy due to lack of food intake". In other words, I speculated that hibernation phase serum is not "rich enough" for cell culture. Hence, in another experiment, I tested both ABSM and HBSM on bears' ADSCs. Interestingly, treatment of a bear ADSCs with both serum types of the same 8 bears (4 ABSM and 4 HBSM) indicated significant cell growth, and formation of spindle-like mesenchymal cell (Fig 1–4, 1–5). I also ruled out the effect of culture dish where I used 35 mm collagen-coated dish for both PBMCs and ADSCs culture. These findings indicate that HBSM is "not poor" for ADSCs culture; however, HBSM (unlike ABSM) fails to promote PBMCs culture.

Previous investigations on bear adipocyte culture by active and hibernating bear serum represented metabolic profiles similar to *in-vivo* (Rigano et al., 2017). In addition, bears' ADSCs have shown optimal

differentiation to different cell lineages such as chondroblasts and osteoblasts (Fink et al., 2011; Gehring et al., 2016). Human osteoblasts that were cultured with bear serum proliferate well (Overstreet et al., 2003, 2004). Furthermore, the osteoblasts show similar response to hibernation and active bear serum (Overstreet et al., 2003, 2004). Altogether, it can be deduced that while hibernating phase serum hinders OC formation, it retains the proliferative and differentiating potential for ADSCs and osteoblasts. This may, in part, participate in hibernating bears' bone mass maintenance.

Still, it remains questionable how hibernating phase serum can hinder osteoclastogenesis, and which serum factors or mechanisms are involved in this phase. Studies on hibernating bears have denoted that there are various serum factors that play roles in bone metabolism. For instance, i) Parathyroid hormone (PTH) is thought to have major anabolic effects on bone metabolism in American black bears (*Ursus americanus*) (Doherty et al., 2014; Donahue et al., 2006). Gray et al. (2012) reported that treatment of dystrophin-deficient mice with black bear's PTH decreased osteoclastic surface and increased osteoblastic surface on the bone, leading to significantly higher hind limb bone density than control group (Gray et al., 2012). ii) It has been reported that melatonin concentration during hibernation is about 7.5 times higher than summer active phase in brown bears (*Ursus arctos*) (Ware et al., 2013). Melatonin has indicated inhibitory effects on osteoclastogenesis *in-vitro*. This was partially attributed to the increased osteoprotegerin: RANKL ratio, by inhibiting RANKL expression in osteoblasts (Suzuki et al., 2002; Cardinali et al., 2003; Maria et al., 2018). iii) It is also possible that serum factors affect bone metabolism indirectly through the modulation of circadian rhythm. Jansen et al. (2016) showed when bear's fibroblasts were cultured with active and hibernation phase serum containing medium, cell molecular rhythm was akin to those of active and hibernation phases, respectively. Circadian rhythm can govern OC activity and formation through different pathways, such as: 1) Regulation of aryl hydrocarbon receptor nuclear translocator-like (Bmal1) in OC (Xu et al., 2016), 2) Modulation of Bmal1 in osteoblasts which leads to alteration in RANKL expression (Takarada et al., 2017), and 3) β-adrenergic and glucocorticoid signaling in OC (Fujihara et al., 2014; Kondo et al., 2015). iv) Black bears' serum immune related factors are altered during hibernation, some of which are thought to affect bone metabolism, e.g. α2-HS-glycoprotein (AHSG), which is an osteogenic inhibitor, is downregulated in winter dormancy (Chow et al., 2013).

The present study for the first time revealed the influence of HBSM on osteoclastogenesis. There are more serum factors than stated above, which are likely to be involved in hampering OC formation/activity, and general bone metabolism (Donahue et al., 2006; Doherty et al., 2014). These demand very large-scale tests to examine the efficacy of the serum factors on OC progenitors. In my preliminary tests, I examined the heat inactivation of bear serum on PBMCs culture. These tests failed to show any differentiation in PBMCs culture either with heat-inactivated ABSM or heat-inactivated HBSM. However, when heat-inactivated ABSM and HBSM were applied to ADSCs, the cell growth was comparable with normal (unheated) ABSM and HBSM. It has been suggested that the failure in cell culture with heat inactivation of serum might be due to some cell lineages (Giard, 1987). It is noteworthy that bone remodeling includes bone resorption and bone formation, undertaken by osteoclasts and osteoblasts, respectively. Former studies suggested that both bone formation and resorption are equally suppressed in hibernating bears; an equilibrium which sustain bone remodeling at a low rate in order to save energy for a long-term torpor (Floyd and Nelson, 1990; Seger et al., 2011; Fedorov et al., 2012; McGee–Lawrence et al., 2015). The present study shed light on OCs, indicating that bears PBMCs under the influence of HBSM fail to form OC in *in-vitro* condition. Osteoclastogenesis markers such as β3 integrin, cathepsin K, TRAP, and

calcitonin are required to be explored regarding bears PBMCs differentiation in the future studies. It must be noted that, PBMCs are not the only source for OC formation *in-vivo*, i.e. bone marrow monocytes, and bone macrophages also account for osteoclastogenesis and bone resorption (Alexander et al., 2011; Tevlin et al., 2014; Batoon et al., 2017; Singleton et al., 2019). Therefore, the effect of HBSM on other OC progenitors remains to be explored. Moreover, further investigations are required to demonstrate biochemical alterations that regulate osteoclastogenesis during winter dormancy.

The evolutionary trends and physiological attributes that enable hibernating mammals such as bears (Floyd and Nelson, 1990; Pardy et al., 2004), ground squirrels (McGee-Lawrence et al., 2011), marmots (Wojda et al., 2012), and woodchucks (Doherty et al., 2012) to preserve bone mass deserve to be explored. In comparison with other hibernating mammals, bears have a large body size, near-to-normal body temperature during hibernation, and a drastic pre-hibernation fat storage (Tsubota et al., 2008a,b); such peculiar differences may bring about bone preservation mechanisms that other hibernating mammals do not possess. McGee-Lawrence (2011) stated that although hibernating ground squirrels preserve macrostructural cortical bone geometry and strength, they undergo trabecular, and cortical bone loss on a microstructural scale during hibernation, which is different from hibernating bears bone condition (McGee-Lawrence et al., 2011). Such differences might be due to species-specific osteogenic alterations, which have also been reported in other mammals. For instance, a transient physiological osteoporosis due to a rapid antler osteogenesis may occur in cervids, which is a distinct feature in comparison with other artiodactyls (Nasoori, 2020a). Hence, comprehensive and comparative studies on mammalian physiological bone preserving/or osteoporotic mechanisms are needed to provide insights into mammalian bone biology.

Chapter 2

Hibernating bear serum results in normal osteogenic differentiation *in-vitro*

Abstract:

Background: Although bears undergo long-term disuse and lack of food intake during hibernation, they do not suffer from osteoporosis. In chapter 1, it was indicated that hibernating bear serum hindered osteoclast formation *in-vitro* (Nasoori et al., 2020). Still, it is not clear whether hibernating bear serum has discouraging or encouraging effects on osteogenic differentiation *in-vitro*.

Materials and methods: Adipose tissue and blood serum were collected from anesthetized adult Japanese black bears (*Ursus thibetanus japonicas*) in active and hibernation season. Adipose tissue derived stem cells (ADSCs) of 3 bears were isolated, and were cultured in osteogenic medium with 10% active bear serum containing medium (ABSM) (n = 7) or hibernating bear serum containing medium (HBSM) (n = 7). Osteogenic differentiation was assessed by Alizarin Red stain, alkaline phosphates (ALP) stain, and Sirius Red stain on days 14, 24, and 34.

Results: Alizarin Red stain showed a continuous mineralization throughout the intervals with similar pattern for both ABSM and HBSM groups (p-value > 0.05). ALP stain in both groups showed a similar pattern; increasing trends up to day 24, and decreasing trends up to day 34 (p-value > 0.05). Although the pattern of results for Sirius Red stain was irregular, it demonstrated collagen synthesis in both groups and all intervals.

Conclusions: I found that osteogenic differentiation in ABSM and HBSM groups was similar *in-vitro*, suggesting an ongoing bone formation in hibernation period, consistent with previous studies in hibernating bears. Therefore, hibernating bear serum leads to osteogenic differentiation.

Introduction:

Hibernating bears do not have physical activity nor food intake for about 4 to 5 months in cold season. Despite such harsh conditions, in which humans and other mammals develop severe bone loss, hibernating bears do not suffer from musculoskeletal disorders (Floyd et al., 1990; Pardy et al., 2004; McGee-Lawrence et al., 2008, 2009). In this respect, cellular response of hibernating bears has largely remained unknown. In chapter 1, it was stated that hibernating bear serum hinders osteoclastogenesis *in-vitro*, being an indicator of reduced bone resorption (Nasoori et al., 2020). Nevertheless, bone formation in *in-vitro* has not been extensively explored in bears (Fink et al., 2011; Gehring et al., 2016). Accordingly, I aimed to evaluate osteogenic differentiation of bears' stem cells cultured with bears' serum.

Serum content is affected by physical activity (Kujala et al., 2013; Beavers et al., 2014) and/or osteoporosis (Minisola et al., 2002; Hein et al., 2003). The use of osteoporotic individuals' serum in cell culture can elicit osteoporotic cell response *in-vitro* (Stringer et al., 2007; Li et al., 2015). Also, the use of hibernating bear serum in cell culture can elicit hibernation-like cell response *in-vitro* (Jansen et al., 2016; Chanon et al., 2018), which is why I implemented bears' serum for peripheral blood mononuclear cells (PBMCs) culture in chapter 1 (Nasoori et al., 2020), and adipose derived stem cells (ADSCs) culture in the present chapter.

ADSCs as well as bone marrow stem cells (BMSCs), are considered efficient multi-potent cells differentiating to a variety of cell lineages such as osteoblasts, chondroblasts, adipocytes, and fibroblasts (Niemeyer et al., 2007; Grottkau and Lin, 2013). Practically, ADSCs have some advantages over BMSCs, e.g. adipose tissue collection is much less invasive, and less costly, while is more accessible than bone marrow specimens, particularly in wild animals such as bears. Previous works have demonstrated that brown bears' (*Ursus arctos*) ADSCs exert multi-potency, and can undergo osteogenic differentiation (Fink et al., 2011; Gehring et al., 2016). However, the likely contrastive effects of hibernation and active phases' serum on osteogenic differentiation have not been elucidated *in-vitro* yet. To my knowledge, this is the first study demonstrating osteogenic differentiation of black bears' ADSCs where cells are cultured in osteogenic medium together with hibernating bear serum containing medium (HBSM) or active bear serum containing medium (ABSM) for 34 days.

I used three different assays including Alizarin Red stain, alkaline phosphatase (ALP) stain, and Sirius Red stain, which demonstrate mineralization, osteoblast differentiation, and collagen synthesis, respectively (Chen et al., 2007; Xu et al., 2007; Hwang and Horton, 2019).

Materials and Methods:

Sampling from animals

Along with sampling addressed in chapter 1, adipose tissue from subcutaneous inguinal and sternal areas was biopsied, and blood was collected from jugular vein of anesthetized adult Japanese black bears (*Ursus thibetanus japonicus*) at Ani Mataginosato Bear Park (Kuma Kuma En) located in Akita Prefecture, Japan, in active and hibernation seasons. After sampling, the animals recovered, and resumed their natural status. Adipose tissue and blood serum were delivered to the lab as described in chapter 1 (Nasoori et al., 2020). The sampling and practice were confirmed by the Committee of Hokkaido University (Permit Number: 18–0179). Bear sample information are summarized in Table 2–1.

Table 2–1.
Bear information, sample type, and collection season.

Bears ID	Type of sample	Season	Age* (year)	Body mass* (kg)
A	ADSCs	August	7	128
B	ADSCs	July	22	113.5
C	ADSCs	July	18	143
D	Serum	May	24	107
A	Serum	August	7	128
E	Serum	May	3	92
F	Serum	May	16	145.5
G†	Serum	May	14	100.5
H†	Serum	May	14	74
I	Serum	May	10	153
A	Serum	January	7	121
J	Serum	January	7	133
F	Serum	January	17	164
K	Serum	January	17	142.5
L	Serum	January	16	196
G†	Serum	January	16	87
H†	Serum	January	16	106

All samples were collected from male bears, except G and H female bear indicated by†. May, July and August samples are for active phase. January samples are for hibernation phase. The average of active and hibernating bears' age (for serum samples) is 12.5 ± 2.5 and 13.7 ± 1.7, respectively. The difference between the groups' age is not significant; *p*-value = 0.75, analysed by two-tailed Student's *t*-test. *Body mass and age at sampling season. ADSCs: Adipose derived stem cells.

ADSCs isolation

ADSCs were isolated as described in chapter 1. Briefly, adipose tissue was digested (1) enzymatically; by collagenase along with BAS in DMEM medium, and (2) mechanically; on a shaker at 37°C for 2 hours. The cells were centrifuged and filtered twice, and finally preserved at -80°C for later uses (Nasoori et al., 2020).

Cell culture

ADSCs were passaged three times. The third passage cells were seeded at a concentration of 7 to 8 $\times 10^4$ cells per well on 24 well plates with a-MEM medium (Minimum Essential Medium Eagle, Sigma-Aldrich, UK) and incubated at 37°C with 5% CO_2. The medium was supplemented with 10% bear serum, 100 units/ml penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporatio), 50 µg/ml gentamicin (FUJIFILM Wako Pure Chemical Corporation), 2.5 µg/ml amphotericin B (FUJIFILM Wako Pure Chemical Corporation) and 2 mM/lit L-Glutamine (Nacalai Tesque Inc., Japan), together with osteogenic factors; 10 nM/lit dexamethasone (Dexamethasone, Sigma-Aldrich, Germany), 200 µM/lit L-Ascorbic acid (Sigma-Aldrich, Germany), and 10 mM/lit β-Glycerophosphate disodium salt hydrate (Sigma-Aldrich, USA). Cells from each of 3 bears (A, B, and C) were cultured separately, and were treated separately with a single type of bear serum as shown in Table 2–2. In total, 7 different ABSM and 7 different HBSM were used.

Table 2–2.
Algorithm of cultured cells from 3 bears with each serum type and staining method for an interval.

	ABSM	HBSM
ADSCs bear A	A E F G H A E F G H A E F G H	A F L G H A F L G H A F L G H
ADSCs bear B	D E H I D E H I D E H I	A J F K A J F K A J F K
ADSCs bear C	D E H D E H D E H	A F K A F K A F K

Cell culture algorithm; each letter under ABSM and HBSM represents a certain type of bear serum used per well. Bear information are summarized in Table 2–1. The present work consists of 3 intervals (days 14, 24, and 34). The above table indicates the cultured cells for only one interval. The same algorithm was implemented for all 3 intervals. At the end of each interval, cells were stained; Red: Alizarin Red stain, Blue: ALP stain, Green: Sirius Red stain. ABSM: active bear serum containing medium. HBSM: hibernating bear serum containing medium. ADSCs: adipose derived stem cells.

Staining procedure

The procedure is the modified version of staining methods explained by Chen et al. (2007), Xu et al. (2007), Hwang and Horton (2019) and the instructions of the company (stated below). The following is about the staining procedure for the cells (per well) cultured in 24 well plates. After removing the medium from the well, cells were very gently washed twice with 600 µl PBS. Next, PBS was removed, and cells were fixed with 300 µl 10% phosphate buffered formalin for 20 minutes. Then, formalin was removed, cells were very gently washed twice with 1 ml distilled (D) water (or with PBS in case of Sirius Red stain). Afterwards, D water/PBS was fully removed, and cells were left for 10 minutes at room temperature to dry. For staining, I used Alizarin Red Staining Solution (Merck KGaA., Germany), which was already pH adjusted (pH 4.1 ± 0.1), filtered with 0.22 µm membrane, followed by addition of 300 µl stain per well. Besides, I used 300 µl ALP (Alkaline Phosphatase Staining Kit, Cosmobio Co., LTD.) and 300 µl Sirius Red stain (Sirius Red Total Collagen Detection Assay Kit, Chondrex, Inc., USA) per well. Stained cells were incubated at 37℃ for 40 minutes, after which were very gently washed twice with D water to stop staining. Cells in each time point and for each treatment were stained as shown in Table 2–2.

Statistical analysis

The stained wells were photographed and analyzed with NIH Image J Software. The data were analyzed with Student's *t*-test, or one-way ANOVA associated with Tukey test and Bonferroni post-hoc test, in which significant *p*-value was considered equal or less than 0.05. The data are represented as average ± SE).

Results:

Alizarin Red stained cells (as the indicator of mineralization/ Ca deposition) showed an increasing trend over 3 intervals (days 14, 24 and 34) and followed a similar pattern in ABSM and HBSM groups. There was a significant difference between day 14 and 34 in HBSM group (*P*-value < 0.05) (Fig 2–1). Other than this, there was not any significant difference between the corresponding days of ABSM and HBSM (Fig 2–1). ALP stained cells (as the indicator of osteoblast differentiation and activity) in ABSM and HBSM groups showed a similar pattern; increasing from day 14 to 24, and decreasing to day 34. There was not any significant difference in each group or between the corresponding days of ABSM and HBSM groups (Fig 2–2). Sirius Red stained cells (as the indicator of collagen synthesis) in both groups exhibited different patterns, but there was not any significant difference in each group or between the corresponding days of the two groups (Fig 2–3).

Regarding total stained areas for 3 bear cells and all 3 intervals (total of day 14, 24, and 34) treated with ABSM and HBSM: Alizarin Red stained cells indicated (average ± SE) 8.16 ± 0.60 and 10.54 ± 1.66, respectively (*p*-value > 0.05), ALP stained cells indicated 3.45 ± 0.64 and 3.82 ± 0.63, respectively (*p*-value > 0.05), and Sirius Red stained cells indicated 43.78 ± 2.28 and 43.34 ± 3.20, respectively (*p*-value > 0.05).

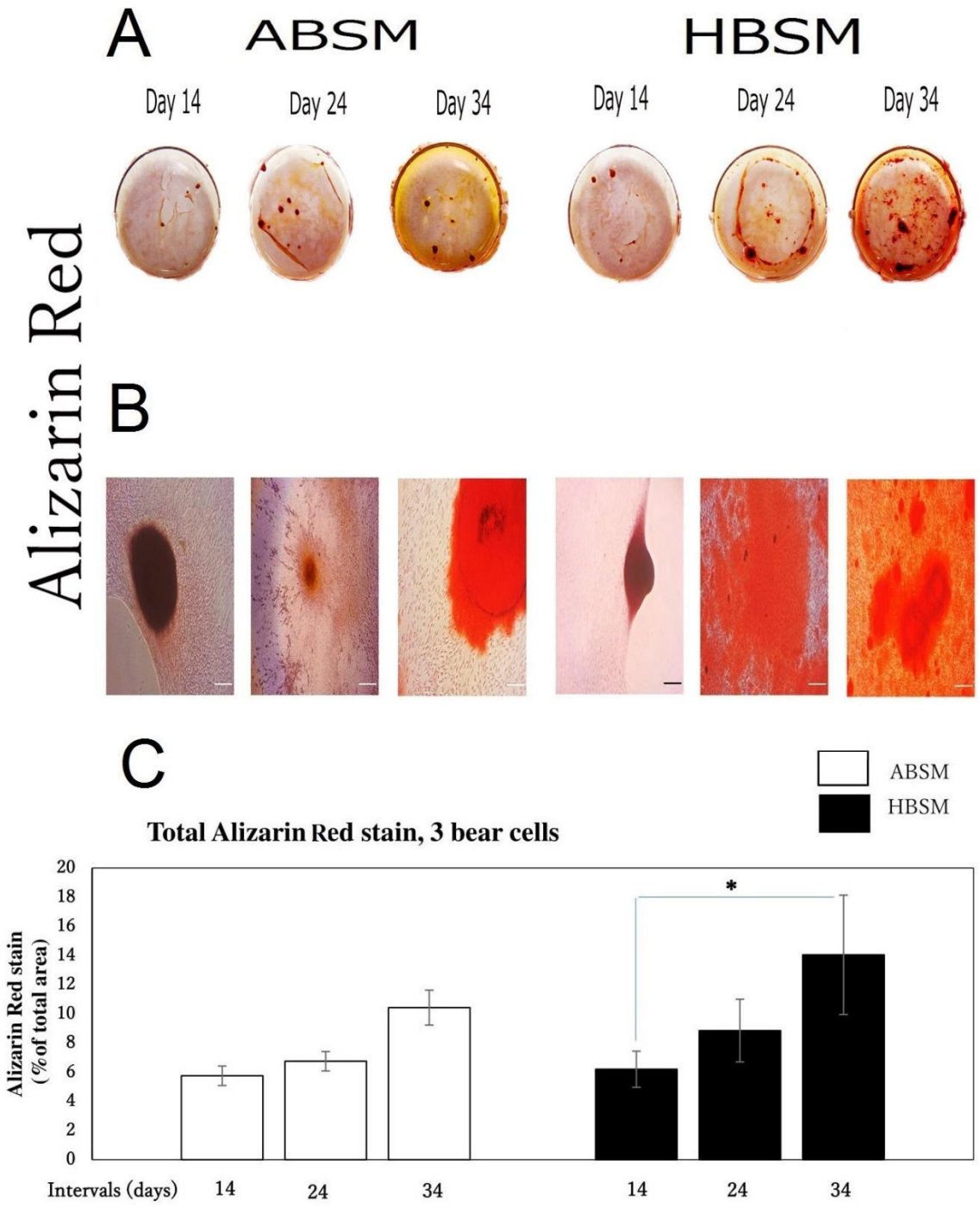

Fig 2–1. Alizarin Red stain. (A) Stained cells on the well cultured with ABSM (active bear serum containing medium) or HBSM (hibernating bear serum containing medium) over 3 intervals. (B) Micrographs of stained cells cultured with ABSM and HBSM over 3 intervals. Scale bar is equal to 200 μm. (C) Each column represents an average of stained area of total of 3 bear cells cultured with 7 different serums of either ABSM or HBSM ± SE. ABSM day 14 = 5.75 ± 0.66, day 24 = 6.75 ± 0.66, day 34 = 10.43 ± 1.19. HBSM day 14 = 6.20 ± 1.23, day 24 = 8.85 ± 2.15, day 34 = 14.04 ± 4.09. * Significant difference (*p*-value = 0.046). No Significant difference in other values (one-way ANOVA).

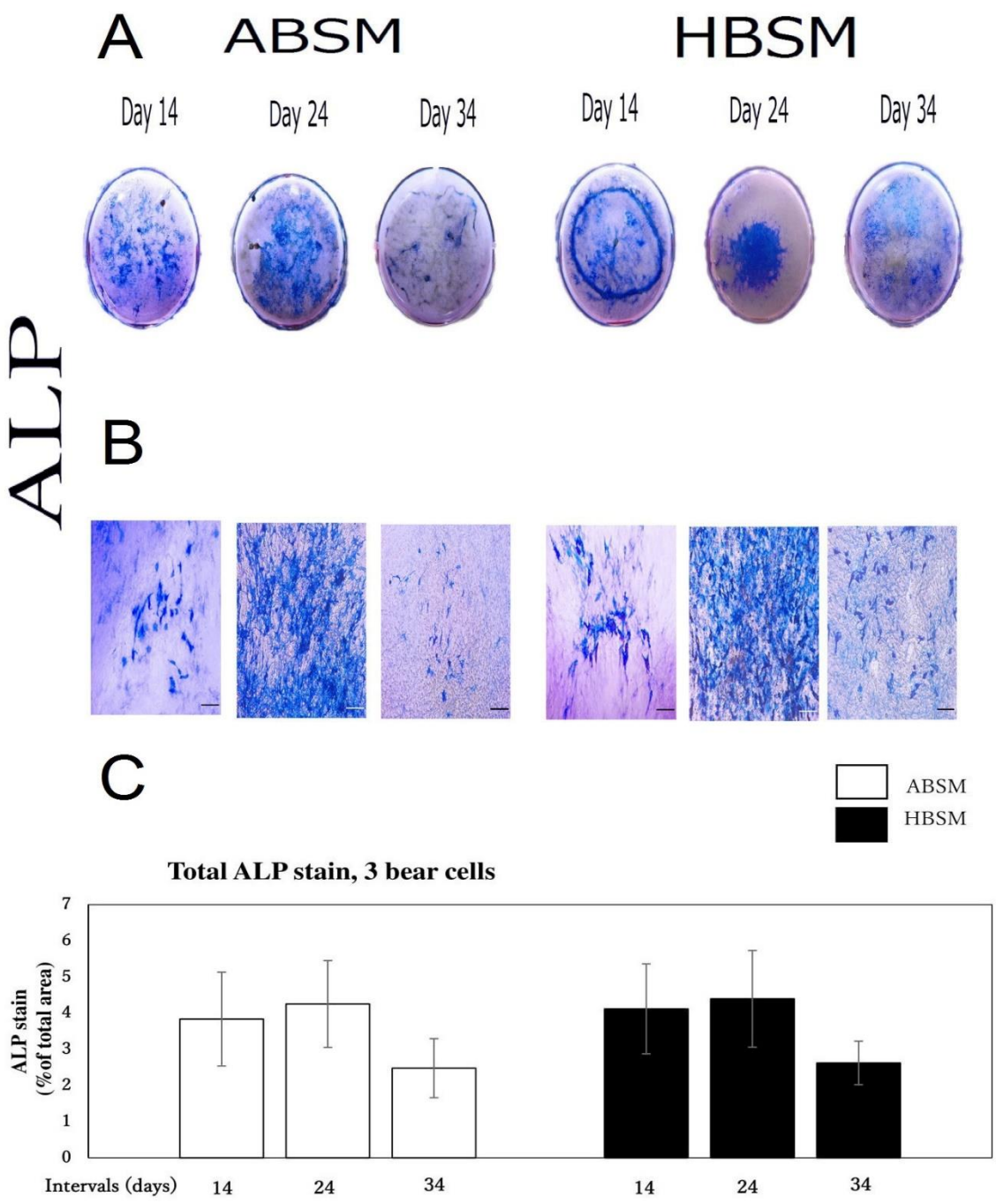

Fig 2–2. ALP stain. (A) Stained cells on the well cultured with ABSM (active bear serum containing medium) or HBSM (hibernating bear serum containing medium) over 3 intervals. (B) Micrographs of stained cells cultured with ABSM and HBSM over 3 intervals. Scale bar is equal to 200 μm. (C) Each column represents an average of stained area of total of 3 bear cells cultured with 7 different serums of either ABSM or HBSM ± SE. ABSM day 14 = 3.83 ± 1.29, day 24 = 4.25 ± 1.19, day 34 = 2.47 ± 0.81. HBSM day 14 = 4.11 ± 1.24, day 24 = 4.39 ± 1.33, day 34 = 2.62 ± 0.60. No significant difference in the represented values (one-way ANOVA).

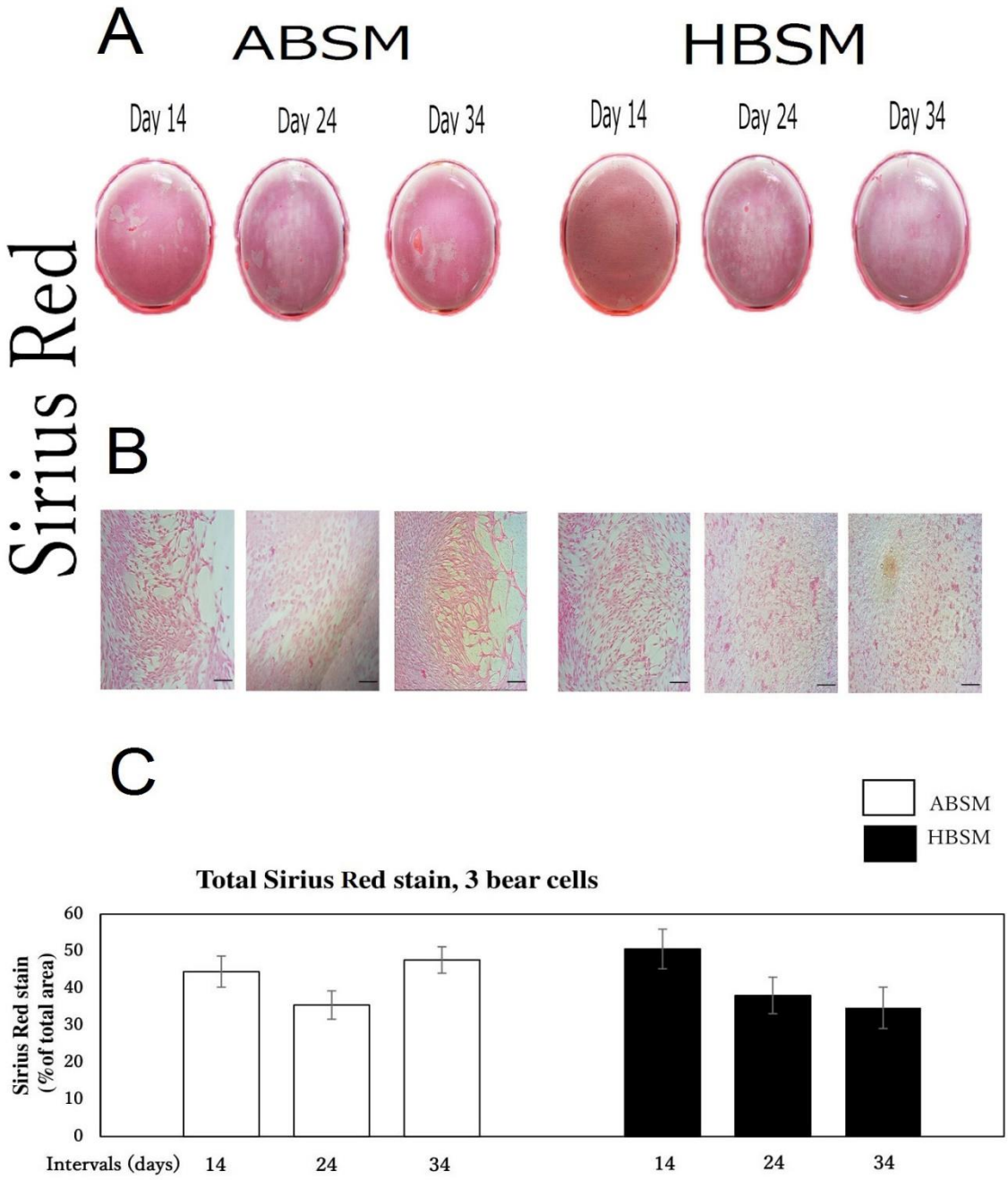

Fig 2–3. Sirius Red stain. (A) Stained cells on the well cultured with ABSM (active bear serum containing medium) or HBSM (hibernating bear serum containing medium) over 3 intervals. (B) Micrographs of stained cells cultured with ABSM and HBSM over 3 intervals. Scale bar is equal to 200 μm. (C) Each column represents an average of stained area of total of 3 bear cells cultured with 7 different serums of either ABSM or HBSM ± SE. ABSM day 14 = 44.48 ± 4.19, day 24 = 35.49 ± 3.82, day 34 = 47.62 ± 3.57. HBSM day 14 = 50.60 ± 5.35, day 24 = 38.07 ± 4.92, day 34 = 34.72 ± 5.57. No significant difference in the represented values (one-way ANOVA).

Discussion:

The current work demonstrated a similar osteogenic pattern between ADSCs cultured with HBSM and ABSM, suggesting a sustained bone formation in hibernating bears. This is consistent with previous studies which unveiled ongoing bone formation in hibernating bears, i.e. bone mass is preserved in hibernating bears (Floyd et al., 1990; Donahue et al., 2003; McGee-Lawrence et al., 2015). In addition, as stated in chapter 1, ADSCs (cultured in non-osteogenic medium enriched with either HBSM or ABSM) exerted similar proliferation and differentiation for two consecutive passages (Nasoori et al., 2020). When ADSCs were cultured in serum free medium, they showed very poor proliferation and differentiation, implying that bears' serum (ABSM and HBSM) has impact on ADSCs differentiation (Nasoori et al., 2020).

The findings in chapter 1 and 2 denote that hibernation phase serum (HBSM) may encourage osteogenic differentiation, while discourage osteoclastic differentiation. The differential physiological or cellular response are not uncommon in hibernating bears, for example i) while metabolic rate is diminished to 25% of active phase, the body temperature remains close to normal in hibernating bears (Tøien et al., 2011), and ii) while adipocytes (adipose tissue) undergo a vigorous catabolic phase, myocytes (muscle mass) retain their protein content close to normal rate during hibernation. Such differences are, in part, attributed to upregulation or downregulation of certain genes during hibernation (Shimozuru et al., 2012, 2016; Miyazaki et al., 2019). Upregulation of certain anabolic genes such as *Fn1*, *Igf1r* and *Sox9* during hibernation was proposed to be involved in bone formation in torpid bears (Fedorov et al., 2012). Similar mechanisms may participate in the dual effects of HBSM. In chapter 1, I discussed the likely mechanisms playing roles in osteoclastogenesis during hibernation phase (Nasoori et al., 2020). Bear parathyroid hormone accounts as a strong bone anabolic (osteogenic) factor (Gray et al., 2012). In addition, vit D2 (25OHD2) to vit D3 (25OHD3) ratio is significantly higher in hibernating bears (Vestergaard et al., 2011). Interestingly, 25OHD2, in comparison with 25OHD3, has significantly less effect on 1,25OHD synthesis, which regulates calcium uptake (Holliday et al., 2000; Lehmann et al., 2013; Shieh et al., 2016; Wilson et al., 2016). In addition, Nelson et al. (2006) suggested that denning (hibernating) bears synthesize a form of vit D3, "24,25-dihydroxyvitamin D3", which has low bone–resorption effect, whereas it can induce bone formation.

I used three different assays to evaluate osteogenic differentiation. Alizarin Red stain demonstrated increasing mineralization trends in both HBSM and ABSM over 34 days of culture (Fig 2–1). These denote that ADSCs steadily give rise to osteogenic cells (such as osteoblasts) mineralizing culture microenvironment. ALP stain demonstrated that ALP expression in both HBSM and ABSM increased from day 14 to day 24, but it decreased on day 34, while the fluctuations were not significant (p-value > 0.05) (Fig 2–2). ALP is an early indicator of osteoblast differentiation and bone formation *in-vitro* and *in-vivo* (Weinreb et al., 1990; Nasoori et al., 2013; Prins et al., 2014). The decrease in ALP level on day 34 (Fig 2–2) might be due to a change in the ratio of the cultured cells, i.e. osteoblasts to osteocytes ratio. Former workers reported that along with increased mineralization, osteoblasts either die or give rise to ultimately differentiated cells; osteocytes (Heino and Hentunen, 2008). Osteocyte formation is usually associated with reduced ALP expression and elevated mineralization in culture microenvironments (Dodds et al., 1993; Schaffler and Kennedy, 2012; Mullen et al., 2013). Such trends in mineralization and ALP activity have been reported in osteogenic differentiation of cells from dogs (Alves et al., 2014),

rodents (Liu et al., 2019), and humans (Fang et al., 2014; Berbéri et al., 2017). Plasma ALP and calcium levels were found similar in active and hibernating captive Asiatic black bears (*Ursus thibetanus*) (Yang et al., 2017). However, McGee-Lawrence et al. (2015) reported a significant reduction in serum bone specific ALP (BSALP) in hibernating American black bears, while calcium level was similar to pre-hibernation and post-hibernation phases. Using serum analysis, McGee-Lawrence et al (2015) deduced that hibernating bears maintain both bone formation and resorption, but at a low rate (reduced bone turnover) due to a general dwindled metabolism to save energy during hibernation.

Sirius Red stained cells existed in all sets of experiments for three intervals in HBSM and ABSM (*p*-valu*e* > 0.05) (Fig 2–3); an indicator of collagen synthesis. Collagen is the most abundant bone protein, which serves crucial functions as matrix in bone structure (Wang et al., 2012; Nair et al., 2013; Nasoori et al., 2013). In the current study, the presence of collagen in culture microenvironment demonstrated osteogenic development. However, the pattern appeared to be not harmonious within the intervals, and appeared to differ from ALP and Alizarin Red stain trends. Such inconsistencies have also been reflected in other works, too, for example a study on osteogenic differentiation of human ADSCs reported lack of ALP expression on day 14 of culture while collagen was apparently synthesized; however, on day 21, ALP expression was associated with a decrease in collagen synthesis (Pre et al., 2011). In another study on osteogenic differentiation of dogs' ADSCs, some inconsistencies appeared between collagen content and ALP/mineralization levels (Alves et al., 2014). Altogether, collagen was present in all of my experimented cells, delineating an ongoing osteogenic differentiation; however, the trend was decreasing in HBSM over time (Fig 2–3). Previous workers, using plasma analysis, suggested that collagen (including collagen type I) is immensely degraded in hibernating brown bears and American black bears (Hissa et al., 1998; Lohuis et al., 2005), and serum collagen is significantly low during hibernation (Vestergaard et al., 2011), which can partially justify decreased Sirius Red values in HBSM culture (Fig 2–3). With regard to cell culture assessment, perhaps it would be better to employ various tests to analyze collagen synthesis, such as immunohistochemistry and mRNA expression tests.

Here, I assessed osteogenic differentiation of stem cells residing in subcutaneous adipose tissue (ADSCs). It is noteworthy that bone marrow adipose tissue content increases in response to the lack of physical activity and/or microgravity (Trudel et al., 2009; Nardo et al., 2013; Keune et al., 2016). Interestingly, bone marrow adipose tissue of hibernating mammals such as marmots (*Marmota flaviventris*) and thirteen line ground squirrels (*Ictidomys tridecemlineatus*), which are resistant to disuse bone loss, increases during hibernation (McGee-Lawrence et al., 2011; Cooper et al., 2016; Doherty et al., 2016). It has been presumed that increased bone adiposity diminishes osteoblast formation and activity. Nevertheless, Keune et al., (2017) reported that bone marrow adipose tissue deficiency increases disuse–induced bone loss in mice, implying that the bone marrow adipose tissue is likely to participate in stabilizing bone turnover (Keune et al., 2017). Thus, osteogenic differentiation of bears' adipose tissue stem cells *in-vitro* can nearly mimic bone formation in hibernating mammals. However, further investigation is required to compare bears' BMSCs and ADSCs osteogenic differentiation *in-vitro*.

Bears' ability to preserve bone mass during hibernation has still remained elusive. Basically, weight-bearing bones (such as long bones) are more liable to undergo disuse osteoporosis (Ruggiu and Cancedda, 2015). However, previous investigators did not find osteopenia in cortical or trabecular structures of hibernating bears' long bones (Floyd et al., 1990; Pardy et al., 2004; McGee-Lawrence et al., 2008, 2009). It must be noted that periosteal bone formation, which plays a crucial role in adult osteogenesis (Morey and Baylink, 1978; Jilka et al., 2009; Debnath et al., 2018), has been scarcely investigated in ursids.

Disuse and unloading considerably reduce periosteal bone formation (Gadomski et al., 2014). In a study on different strains of mice, C3H mice showed resistance to disuse osteoporosis indicated by compensatory role of periosteum in response to unloading–induced endocortical bone loss (Judex et al., 2004). It can be presumed that bears' bone tissue possesses species-specific features to endure disuse and low metabolism. In this respect, for instance, a species-specific osteogenic feature has been reported regarding cervids' antler "specialized periosteum", which confers a unique bone growth rate (~ 2 cm per day) in antlers; a distinct feature among mammals (Li et al., 2014; Nasoori, 2020a). Also, ursids' hibernation has evolved in response to harsh climatic conditions (Shimozuru et al., 2013; Evans et al., 2016). Hard tissues, including skeletal system, have developed degrees of adaptations depending on type of ecosystem (Wang et al., 2017; Nasoori 2020b). Nonetheless, such interactions and compatibilities in skeletal system remain to be further investigated in terms of ursids' physiology and phylogeny.

In conclusion, my study indicates that HBSM exerts osteogenic differentiation similar to that of ABSM in terms of rate and pattern *in-vitro*. This may denote that bone formation is ongoing in hibernating bears, being a mechanism involved in bone preservation during hibernation. Further investigations are required to assess other sources of osteogenic cells, such as bone marrow stem cells and periosteum, by the use of a variety of tests to provide more comprehensive insight into bears' osteogenesis. Also, physiological responses and phylogenic trends, which have enabled ursids to adapt their skeletal system to low metabolism and long-term inactivity, should be further studied.

Conclusion:

Hibernation is an enigmatic physiological response occurring in certain species. Hibernating mammals tend to reduce their metabolic rate in order to save energy in response to low ambient temperature and food scarcity. Unfavorable climatic conditions in cold season make hibernating mammals shelter in their hibernacula for a prolonged period of time. Hibernating bears remain inactive for about 4 or 5 months without food intake, defecation and urination, being distinct among other hibernating mammals. Comparable conditions can lead to severe health issues in other species such as humans. Bears' resistance to disuse osteoporosis has been investigated by different investigators using imaging, histological, and biochemical tests. Accordingly, skeletal system of hibernating bears is not affected by long-term inactivity, or lack of food intake and excretion. These indicate that hibernating bears benefit from a stable homeostatic response throughout the torpor phase.

In the present study, I aimed to know whether the bear serum can cause hibernation-like status in *in-vitro* conditions in which bear cells are cultured with bear serum. For this matter, I used peripheral blood mononuclear cells (PBMCs) and adipose derived stem cells (ADSCs) which undergo osteoclastogenic and osteogenic differentiation *in-vitro*, respectively. The samples were obtained from captive Japanese black bears (*Ursus thibetanus japonicus*) after induction of anaesthesia. After isolation of PBMCs and ADSCs, cells were exposed to appropriate culture medium, as described in chapter 1 and 2.

In chapter 1, I used PBMCs which I isolated from 3 bears. Each bear PBMCs was separately cultured with 10% serum of 4 active and 4 hibernating bears (each individual serum type was assessed separately by a bear PBMCs). PBMCs were cultured in medium with osteoclastogenic factors; macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL). The cultured cells with medium were incubated at 37°C with 5% CO_2 for 11 days. I found that PBMCs cultured with active bear serum fully differentiated to osteoclast-like cells characterized by multinucleated cell morphology and positive tartrate resistant acid phosphatise (TRAP) stain. However, PBMCs cultured with hibernating bear serum did not form osteoclast-like cells and showed significant less TRAP stain than active phase serum group.

In chapter 2, I used ADSCs which I isolated from 3 bears. Each bear ADSCs was separately cultured with 10% serum of 7 active and 7 hibernating bears (each individual serum type was assessed separately by a bear ADSCs). ADSCs were cultured in osteogenic medium and incubated at 37°C with 5% CO_2 for 34 days. I found that osteogenic differentiation revealed by Alizarin Red, ALP, and Sirius Red stains was similar between the cells cultured with active bear serum and hibernating bear serum over three intervals; days 14, 24 and 34.

The overall results in chapter 1 and 2 showed that hibernating bears' serum hindered osteoclast formation, while it exerted normal (similar to active phase serum) osteogenic differentiation, suggesting that hibernating bear serum exert dual effects. These results in *in-vitro* model delineate that bone formation is ongoing, while osteoclast formation is hindered, being a mechanism to preserve bone mass during hibernation. This is consistent with previous works which showed hibernating bears do not suffer from osteopenia and osteoporosis. Also, my results may denote that the use of hibernation phase serum *in-vitro* may result in hibernation-like conditions. The use of *in-vitro* tests will open up new areas for doing research regarding hibernating bears with minimal effects on bears health and welfare.

The findings of the present study highlight the importance of further investigations into bear physiology and homeostatic response during hibernation. Such studies will provide important insights into biological and medical disciplines. Altogether, these findings denote the importance of wildlife as a great source for new discoveries, and indicate the value of wildlife as an asset which must be carefully protected.

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