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Wawrzyniak

mgr Natalia Wawrzyniak

Badanie wpływu wybranych czynników żywieniowych i farmakologicznych na gospodarkę mineralną i metabolizm tkanki kostnej w zwierzęcym modelu osteoporozy pomenopauzalnej

Rozprawa na stopień naukowy doktora nauk rolniczych

w dyscyplinie technologia żywności i żywienia

Promotor: prof. dr hab. Joanna Suliburska

Katedra Żywienia Człowieka i Dietetyki

Wydział Nauk o Żywności i Żywieniu

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za kształtowanie mojej postawy naukowej,

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Dedykuję tę pracę

Mojej Mamie i Synowi Filipowi

Spis treści

Wykaz publikacji włączonych do cyklu

1. Nutritional and health factors affecting the bioavailability of calcium: a narrative review

   Autorzy: Wawrzyniak N., Suliburska J.

   Czasopismo: Nutrition Reviews 2021

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   Punktacja MNiSW: 140

2. Effects of calcium lactate-enriched pumpkin on calcium status in ovariectomized rats

   Autorzy: Wawrzyniak N., Gramza-Michałowska A., Pruszyńska-Oszmałek E., Sassek M., Suliburska J.

   Czasopismo: Foods 2022, 11, 2084

   DOI: 10.3390/foods11142084

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   Punktacja MNiSW: 100

3. Calcium carbonate -enriched pumpkin affects calcium status in ovariectomized rats

   Autorzy: Wawrzyniak N., Gramza-Michałowska A., Kurzawa P., Kołodziejski P., Suliburska J.

   Czasopismo: Journal of Food Science and Technology 2023; 60(4):1402–1413

   DOI: 10.1007/s13197-023-05686-3

   Impact Factor: 3.117

   Punktacja MNiSW: 70

4. Effects of ovariectomy and calcium enriched pumpkin on magnesium status in rats

   Autorzy: Wawrzyniak N., Gramza-Michałowska A., Suliburska J.

   Czasopismo: Acta Scientiarum Polonorum Technologia Alimentaria 2023; 21(4):439–48

   DOI: 10.17306/J.AFS.2022.1108

   Impact Factor: brak

   Punktacja MNiSW: 40

5. Effect of pumpkin enriched with calcium lactate on iron status in an animal model of postmenopausal osteoporosis

   Autorzy: Wawrzyniak N., Gramza-Michałowska A., Suliburska J.

   Czasopismo: Open Chemistry 2023;21:20220314

   DOI: 10.1515/chem-2022-0314

   Impact Factor: 1.977

   Punktacja MNiSW: 70

Sumaryczny Impact Factor: 17.765
Sumaryczna punktacja MNiSW: 420
Sumaryczna liczba cytowań: 21

Wykaz skrótów

AMP - adenosine monophosphate - adenozynomonofosforan

BMD – bone mineral density - gęstość mineralna kości

CaSR - calcium sensing receptor - receptor wykrywający wapń

DMT1 - divalent metal transporter 1 - transporter metali dwuwartościowych

ES – estrogen

HGB – hemoglobin - hemoglobina

OC - osteocalcin - osteokalcyna

PINP – procollagen type-1 amino-terminal propeptide - N-końcowy propeptyd prokolagenu typu I

PTH – parathyroid hormone - parathormon

SCFA – short-chain fatty acids – krótkołańcuchowe kwasy tłuszczowe

Sham – grupa pozornie operowana

Grupy zwierząt :

- **C** - grupa kontrolna - dieta standardowa
- **OVX_C** – grupa owariektomizowana – dieta standardowa
- **DEF** - grupa owariektomizowana - dieta z deficytem wapnia
- **CaC_B** - grupa owariektomizowana - dieta standardowa z dodatkiem alendronianu
- **P_CaC** - grupa owariektomizowana - dieta z dynią wzbogaconą w węglan wapnia
- **P_CaC_B** - grupa owariektomizowana - dieta z alendronianem i dynią wzbogaconą w węglan wapnia
- **CaL** - grupa owariektomizowana - dieta z mleczanem wapnia
- **P_CaL** - grupa owariektomizowana – dieta z dynią wzbogaconą w mleczan wapnia
- **CaL_B** - grupa owariektomizowana – dieta z alendronianem i mleczanem wapnia
- **P_CaL_B** - grupa owariektomizowana – dieta z alendronianem i dynią wzbogaconą w mleczan wapnia

Aktywność naukowa doktorantki

Urodziłam się 30 sierpnia 1991 roku w Poznaniu. Po uzyskaniu podstawowego wykształcenia w 2007 roku rozpoczęłam naukę w VIII Liceum Ogólnokształcącym im. Adama Mickiewicza w Poznaniu. Ukończyłam je w 2010 roku i rozpoczęłam studia stacjonarne na Uniwersytecie Medycznym im. Karola Marcinkowskiego w Poznaniu na kierunku Dietetyka. W 2014 roku uzyskałam tytuł licencjata broniąc pracę dyplomową pt. „Zadowolenie ze swojego ciała a masa ciała młodych kobiet". W 2016 roku rozpoczęłam studia magisterskie na Uniwersytecie Przyrodniczym w Poznaniu na kierunku Dietetyka. Chęć ciągłego zdobywania wiedzy przyczyniła się do wyjazdu w ramach programu Erasmus+ do Justus-Liebig-Universität w Gießen. W ciągu dwóch semestrów uczyłam się na kierunku Ernährungswissenschaften (nauki o żywieniu) w języku niemieckim. Po powrocie do Polski w roku 2018 obroniłam pracę dyplomową pt. „Świadomość konsumencka na temat zastosowania konserwantów w produktach spożywczych". Po zakończeniu studiów magisterskich w październiku 2018 roku wyjechałam na dwumiesięczny staż do działu naukowego firmy Frankenförder Forschungsgesellschaft w Berlinie w ramach programu Erasmus+, gdzie brałam udział w rozwoju produktu i procesu produkcji innowacyjnej żywności, jakim były wegańskie przekąski.

W maju 2019 roku wygrałam konkurs na stypendium naukowe w projekcie OPUS finansowanym ze środków Narodowego Centrum Nauki: „Badania nad możliwością wykorzystania matrycy roślinnej wzbogaconej w wapń w prewencji i terapii osteoporozy", którego kierownikiem była pani prof. dr hab. Anna Gramza-Michałowska. W październiku 2019 roku rozpoczęłam studia trzeciego stopnia w Szkole Doktorskiej na Uniwersytecie Przyrodniczym w Poznaniu. Podczas czteroletnich studiów stacjonarnych moje działania naukowe koncentrowały się na zagadnieniu dotyczącym wpływu dyni wzbogaconej w wapń na gospodarkę mineralną i metabolizm tkanki kostnej w zwierzęcym modelu osteoporozy pomenopauzalnej. Efektem przeprowadzonych badań i analiz jest pięć artykułów naukowych o łącznej punktacji MNiSW - 420 pkt oraz IF – 17.765, które wchodzą w cykl publikacji do pracy doktorskiej.

Moja pozostała działalność naukowo-dydaktyczna podczas studiów w Szkole Doktorskiej wiązała się z prowadzeniem 3-letniego projektu Preludium finansowanym ze środków Narodowego Centrum Nauki pt. „Ocena wpływu equolu na gospodarkę wapnia u szczurów owariektomizowanych" i dwóch projektów w ramach projektu Młoda Kadra

pt. „Badanie związku adipokin i cytokin ze zmianami osteoporotycznymi u szczurów w modelu osteoporozy pomenopauzalnej” oraz „Badanie związku stłuszczenia i zapalenia wątroby ze zmianami osteoporotycznymi u szczurów w modelu osteoporozy pomenopauzalnej”. Brałam również udział w wielu konferencjach związanych z technologią żywności i żywienia.

Od października 2021 biorę udział w projekcie "Zintegrowany Program Uniwersytetu Przyrodniczego w Poznaniu na rzecz Innowacyjnej Wielkopolski". Projekt ma na celu trwałą poprawę jakości i efektywności funkcjonowania Uniwersytetu Przyrodniczego w Poznaniu w aspektach nauczania i zarządzania uczelnią poprzez wdrożenie zintegrowanego programu na rzecz rozwoju regionalnego m.in. poprzez dostosowanie programów kształcenia realizowanych w ramach nauk o żywności do potrzeb społeczno-gospodarczych regionu.

Dodatkowo w lipcu 2022 odbyłam staż w Walencji w Universitat Politècnica de València (Department of Food Technology), aby rozwijać swoje umiejętności w projektowaniu żywności - poznałam nowe techniki rozwoju produktu polegające na zastosowaniu syntezy środków przeciwdrobnoustrojowych opartych na immobilizacji olejków eterycznych na mezoporowatych cząstkach krzemionki.

Powyższe aktywności pozwoliły mi na sukcesywne poszerzanie wiedzy i budowanie dorobku naukowego, na który obecnie składa się 12 anglojęzycznych artykułów opublikowanych w czasopismach z tzw. Listy Filadelfijskiej (w tym artykuły do cyklu pracy doktorskiej), 1 artykuł naukowy opublikowany w recenzowanym polskim czasopiśmie naukowym, a także rozdział w monografii Polskiego Towarzystwa Technologów Żywności pt. „Przyszłość w żywności – żywność w przyszłości” o łącznej punktacji MNiSW – 910 pkt. oraz IF – 35.403.

W czerwcu 2023 zostałam Laureatką Stypendium Miasta Poznania w konkursie dla młodych badaczy z poznańskiego środowiska naukowego.

Omówienie cyklu publikacji

Założenia i cele pracy
wraz z uzasadnieniem połączenia publikacji w cykl

Osteoporoza to choroba charakteryzująca się stopniowym ubytkiem masy kostnej, co prowadzi do osłabienia struktury kości i zwiększenia ryzyka złamań (Black, Rosen, 2016). Osteoporoza pomenopauzalna występuje zwykle ok. 45-55 roku życia. W wyniku zmniejszenia się ilości hormonów żeńskich, zwłaszcza estrogenu (ES), dochodzi do stopniowej utraty masy kostnej. Osteoporoza jest niebezpieczną chorobą, ponieważ zwykle nie daje żadnych objawów przed złamaniem, a te mogą wystąpić nawet przy niewielkim urazie lub podczas wykonywania codziennych czynności (Watts i in., 2021). Szacuje się, że osteoporoza dotyka ok. 200 milionów kobiet na całym świecie, natomiast u 1 na 3 kobiety po 50. roku życia dochodzi do złamania kości z powodu osteoporozy (Anam, Insogna, 2021). Osteoporoza pomenopauzalna jest chorobą, która ma poważny wpływ na jakość życia kobiet, ograniczając ich aktywność i zwiększając ryzyko chorób i powikłań (Anupama i in., 2020). Niska gęstość mineralna kości może być leczona farmakologicznie poprzez stosowanie doustnych leków, np. alendronianu - leku należącego do grupy bifosfonianów (Wang i in., 2017). Jednak długotrwałe stosowanie leków prowadzi do licznych skutków ubocznych (Radwan i in., 2022). Dlatego też terapię osteoporozy należy wspierać również w sposób niefarmakologiczny poprzez aktywny tryb życia, utrzymywanie prawidłowej masy ciała oraz wystarczającą podaż wapnia i witaminy D (Kanis i in., 2020).

Wapń jest głównym składnikiem mineralnym kości, dlatego odpowiednia jego podaż jest ważnym czynnikiem w prewencji i terapii osteoporozy. Występuje w kościach w postaci hydroksyapatytu [$Ca_{10}(PO_4)_6(OH)_2$] - około 99% całkowitego wapnia w organizmie występuje w kościach (Murshed, 2018). Odbudowa kości, czyli wymiana tkanki na nową, następuje przez całe życie; jednak w okresie menopauzy resorpcja kości dominuje nad kościotworzeniem, co prowadzi do znacznego obniżenia gęstości mineralnej kości (BMD) (L. Song, 2017).

Podaż wapnia u kobiet po menopauzie powinna wynosić 1200 mg dziennie (Jarosz i in., 2020). Jednak niedobór wapnia stanowi problem na całym świecie. Populacje w niewielu krajach charakteryzują się wystarczającym średnim spożyciem. Najniższe

średnie spożycie obserwuje się w Azji (<400–500 mg dziennie), średnie spożycie na poziomie 400–700 mg/dobę odnotowuje się w Afryce i Ameryce Południowej, natomiast spożycie wapnia powyżej 1000 mg/dobę obserwowane jest w krajach północnej Europy, z najwyższą średnią podażą w Islandii, wynoszącą 1233 mg/dobę (Balk i in., 2017).

Skutecznym i łatwym sposobem na zwiększenie ilości wapnia w diecie jest zastosowanie żywności wzbogaconej w wapń (Weaver, Liebman, 2002). Jednym z takich innowacyjnych produktów jest dynia wzbogacona w wapń poprzez odwodnienie osmotyczne przy użyciu inuliny (Kulczyński i in., 2021). Dynia zawiera szereg związków bioaktywnych, które sprzyjają zwiększeniu gęstości mineralnej kości. Jednym z nich jest luteina, która zwiększa masę mineralną kości, jednocześnie hamując ich resorpcję poprzez hamowanie tworzenia osteoklastów (Takeda i in., 2017; Tominari i in., 2017) oraz zmniejszenie stanu zapalnego (Li i in., 2018). Dynia zawiera też β-kryptoksantynę, która działa osteogennie poprzez wpływ na ekspresję genów białek biorących udział w tworzeniu kości (Yamaguchi, 2012). Oprócz wpływu na metabolizm kości, dynia wykazuje działanie hipoglikemiczne i kardioprotekcyjne, dlatego jej spożywanie jest zalecane osobom z nadciśnieniem, otyłością i cukrzycą. Dynia jest niskokaloryczna (ok. 26 kcal/100g), a ponadto jest dobrym produktem do przygotowania dań zarówno dla niemowląt, osób starszych, pacjentów z chorobami przewodu pokarmowego ze względu na łatwość zmiany konsystencji (Kulczynski, Gramza-Michałowska, 2019). Zastosowanie inuliny jako substancji osmotycznie czynnej również może poprawiać zdrowie kości w wyniku zwiększenia wchłaniania wapnia czy hamowania ekspresji czynników prozapalnych (Qin i in., 2023).

Metabolizm wapnia jest powiązany z gospodarką innych składników mineralnych, takich jak magnez i żelazo. Magnez jest zaangażowany w hydroksylację witaminy D do jej aktywnej formy ($1,25(OH)_2D$), przez co wpływa na absorpcję wapnia (Rosanoff i in., 2016), natomiast witamina D stymuluje wchłanianie magnezu (Lips, 2012). Niedobór magnezu skutkuje upośledzoną reakcją parathormonu (PTH) (Uwitonze, Razzaque, 2018), który bierze udział w metabolizmie wapnia. Ponadto magnez wpływa na aktywny transport jonów wapnia przez błonę komórkową, a wapń ma kluczowe znaczenie w skurczu mięśni, przewodzeniu impulsów nerwowych, prawidłowy rytm serca i napięcie naczynioruchowe (Gröber i in., 2015). Magnez wpływa na metabolizm wapnia i wiadomo, że suplementacja tych dwóch składników mineralnych jest dodatnio skorelowana z BMD u kobiet po menopauzie (Mahdavi-Roshan i in., 2015; Mutlu i in., 2007). Wapń może też hamować

wchłanianie żelaza poprzez wpływ na transporter metali dwuwartościowych (DMT1) lub hamować przenoszenie jonów żelaza do krwi. Wyniki badania wskazują jednak na krótkotrwały efekt tego działania (Lönnerdal, 2010). Badając wpływ żywności wzbogaconej w wapń na organizm należy zatem kontrolować również gospodarkę magnezu i żelaza.

Cel główny: Celem pracy było określenie wpływu wybranych czynników żywieniowych i farmakologicznych na gospodarkę mineralną i metabolizm tkanki kostnej w zwierzęcym modelu osteoporozy pomenopauzalnej.

Cel ten jest realizowany w oparciu o następujące **cele szczegółowe:**

1. Ocena wpływu dyni wzbogaconej w węglan i mleczan wapnia na gospodarkę wapnia i metabolizm tkanki kostnej u szczurów po owariektomii.
2. Badanie działania dyni wzbogaconej w wapń oraz soli wapnia w połączeniu z alendronianem na gospodarkę wapnia i zdrowie kości u szczurów z usuniętymi jajnikami.
3. Określenie wpływu dyni wzbogaconej w wapń na stężenie magnezu i żelaza w organizmie szczurów po owariektomii.

Materiały i metody

Wzbogacanie dyni w wapń: Tkanki dyni zostały wzbogacone solą wapnia w procesie odwadniania osmotycznego przy pomocy inuliny - substancji osmotycznie czynnej. Podczas odwadniania osmotycznego następuje wymiana składników między roztworem a dynią; z dyni usuwana jest woda, a rozpuszczone w roztworze związki (inulina i węglan lub mleczan wapnia) przenikają do tkanki dyni. Celem odwadniania osmotycznego dyni było nasycenie jej tkanek wapniem i inuliną, tak aby stała się źródłem tych składników.

Najpierw dynię umyto i oczyszczono, a następnie usunięto wewnętrzną część wraz z pestkami. Następnie usunięto skórę, a miąższ dyni pokrojono w sześciany (1 cm), które następnie poddano odwodnieniu osmotycznemu. Przed kolejnym etapem dynia została zamrożona −18◦C i przechowywana przez 24 godziny do dalszej analizy. Przygotowano 50% roztwór inuliny w małych słoiczkach zawierających 125 ml wody destylowanej i 125 g inuliny, dodano sól wapnia, aby uzyskać stężenie 5%. Do tego hipertonicznego

roztworu dodano zamrożone kostki dyni w stosunku 1:5 (50 g dyni i 250 g roztworu); słoiki szczelnie zamknięto i wytrząsano w łaźni wodnej nagrzanej do temp. 50◦C przez 2 godz. Po odwodnieniu osmotycznym usunięto supernatant i dynię przesączono. Całą procedurę wykonano w trzech powtórzeniach. Przed procesem liofilizacji dynia została zamrożona w temp. pomiędzy −18◦C i −28◦C przez 24 godz. Odsączoną dynię suszono w liofilizatorze do zawartości wody 3,5–5%. Następnie liofilizat zmielono i dodawano do karmy dla szczurów.

Badanie na zwierzętach: Sto 12-miesięcznych szczurów (Szczur Wędrowny, Rattus norvegicus, stado niekrewniacze, Wistar) zostało pozyskanych z Wielkopolskiego Centrum Zaawansowanych Technologii Uniwersytetu im. Adama Mickiewicza (Poznań, Polska). Badanie uzyskało zgodę Lokalnej Komisji Etycznej (nr 34/2019). Wszystkie szczury karmiono dietą AIN-93M. Zwierzęta podzielono losowo na dziesięć grup po 10 szczurów. Na początku eksperymentu całkowita masa ciała szczurów nie różniła się między grupami. 90 szczurom usunięto jajniki (OVX), aby stworzyć szczurzy model osteoporozy pomenopauzalnej. Po 7 dniach rekonwalescencji rozpoczęto interwencję żywieniową trwającą 12 tygodni. Grupa kontrolna (C) i jedna z grup z wyciętymi jajnikami (OVX_C) otrzymywały standardową dietę (bez modyfikacji), grupa DEF otrzymywała dietę z deficytem wapnia, grupa CaC_B otrzymywała dietę standardową z dodatkiem alendronianu (lek z grupy bifosfonianów), grupa P_CaC dostawała dietę z dynią wzbogaconą w węglan wapnia, grupa P_CaC_B otrzymywała dietę z alendronianem i dynią wzbogaconą w węglan wapnia, grupa CaL była karmiona standardową dietą z mleczanem wapnia, grupa P_CaL była karmiona dynią wzbogaconą w mleczan wapnia, grupie CaL_B podawano alendronian i mleczan wapnia, a grupie P_CaL_B - alendronian i dynię wzbogaconą mleczanem wapnia. Wszystkie diety (z wyjątkiem DEF) zapewniały taką samą ilość wapnia jak w diecie standardowej (0,5%). W dietach z bifosfonianami ilość alendronianu korygowano co tydzień, aby utrzymać dawkę 3 mg na kilogram masy ciała. Zwierzętom pozwolono jeść i pić wodę dejonizowaną ad libitum przez cały czas trwania doświadczenia. Szczury w każdej grupie ważono co tydzień i codziennie rejestrowano spożycie pokarmu. Po zakończeniu doświadczenia przeprowadzono analizę składu ciała wszystkich zwierząt na analizatorze składu ciała Bruker LF90II. Następnie szczury w każdej grupie dekapitowano i pobierano krew i tkanki. Krew odwirowano przy 1200×G przez 10 minut w 4◦C. Wątroby, śledziony, nerki, trzustki, kości udowe, mięśnie udowe i sierść usunięto, przemyto solą fizjologiczną, zważono i przechowywano w temp. −80◦C

do analizy. W surowicy oznaczono stężenie N-końcowego propeptydu prokolagenu typu I (PINP), PTH, ES i osteokalcyny (OC) za pomocą testu immunoenzymatycznego Elisa. Przeprowadzono analizę morfologiczną krwi pełnej. Stężenie wapnia w surowicy oraz zawartość wapnia, magnezu i żelaza w tkankach zostały określone metodą spektrofotometrii atomowo-absorpcyjnej po uprzedniej mineralizacji.

Uzasadnienie połączenia publikacji w cykl

W pracy "**Nutritional and health factors affecting the bioavailability of calcium: a narrative review**" dokonano przeglądu aktualnych doniesień naukowych dotyczących wpływu czynników żywieniowych i zdrowotnych na biodostępność wapnia. Niniejsza praca stanowi szczegółowy przegląd piśmiennictwa i jest odzwierciedleniem aktualnego stanu wiedzy związanego z powyższym zagadnieniem. Ponadto, kompleksowe usystematyzowanie dostępnych doniesień naukowych umożliwiło identyfikację problemów badawczych, które wymagają dalszych rozwiązań. Artykuł podkreśla istotność unikania czynników ograniczających biodostępność wapnia jak i zwiększania czynników, które sprzyjają wchłanianiu tego składnika mineralnego. Wniosek ten stał się podstawą do przeprowadzenia badań składających się na niniejszą rozprawę doktorską.

W pracy "**Effects of calcium lactate-enriched pumpkin on calcium status in ovariectomized rats**" zbadano wpływ soli organicznej (mleczanu wapnia) na metabolizm wapnia w zwierzęcym modelu osteoporozy pomenopauzalnej. W tym celu zastosowano dietę standardową z dodatkiem mleczanu wapnia, dynię wzbogaconą mleczanem wapnia oraz kombinację wzbogaconej dyni z alendronianem i mleczanu wapnia z alendronianem. W pracy określono wpływ diet modyfikowanych na zawartość wapnia w surowicy i tkankach oraz stężenie PTH, PINP, OC i ES w surowicy u szczurów po owariektomii.

Celem pracy „**Calcium carbonate-enriched pumpkin affects calcium status in ovariectomized rats**" było zbadanie wpływu soli nieorganicznej wapnia (węglanu wapnia) na metabolizm wapnia w zwierzęcym modelu osteoporozy pomenopauzalnej. Podobnie jak w poprzedniej pracy, w karmieniu szczurów zastosowano wzbogaconą dynię, dietę standardową z alendronianem oraz połączenie wzbogaconej dyni z lekiem, z tym że mleczan wapnia został zastąpiony węglanem wapnia. Węglan wapnia jest solą nieorganiczną najczęściej stosowaną w suplementach dla kobiet po menopauzie. W badaniu określono stężenie wapnia w surowicy i tkankach oraz oznaczono parametry

metabolizmu wapnia, dokonano również analizy histopatologicznej kości udowych, gdzie określono liczbę komórek kości, stopień stłuszczenia szpiku oraz procentową zawartość kości woven.

W tkankach szczurów po owariektomii oznaczono również zawartość magnezu i żelaza, a wyniki stały się podstawą do napisania dwóch kolejnych artykułów.

Celem pracy „**Effects of ovariectomy and calcium enriched pumpkin on magnesium status in rats**" było określenie wpływu dyni wzbogaconej w mleczan wapnia na zawartość magnezu w tkankach, natomiast w artykule „**Effect of pumpkin enriched with calcium lactate on iron status in an animal model of postmenopausal osteoporosis**" oceniono działanie dyni wzbogaconej w mleczan wapnia na stężenie żelaza w tkankach u szczurów po owariektomii.

W swojej pracy doktorskiej zdecydowałam się na zaprezentowanie prac związanych z działaniem tylko jednej soli - mleczanu wapnia na gospodarkę magnezu i żelaza, gdyż wyniki wskazują na podobny wpływ obu soli (organicznej i nieorganicznej). Wyniki dotyczące wpływu węglanu wapnia na stężenie magnezu i żelaza w organizmie szczurów przedstawię w kolejnej planowanej publikacji.

Omówienie osiągnięć badawczych kandydata opisanych w cyklu publikacji na tle aktualnego stanu wiedzy

W pracy „**Effects of calcium lactate-enriched pumpkin on calcium status in ovariectomized rats"** wykazano, że grupa P_CaL miała znacznie wyższą zawartość wapnia w surowicy niż grupa CaL. Owariektomia nieznacznie obniżyła zawartość wapnia w kościach udowych, podczas gdy grupa P_CaL_B wykazała jego znaczny wzrost. Wzbogacona dynia ponad dwukrotnie zwiększyła stężenie wapnia w nerkach w porównaniu z grupami C i OVX_C, a alendronian – sześciokrotnie. Zaobserwowano też, że owariektomia zwiększyła stężenie PINP w surowicy, a diety modyfikowane go obniżyły, z wyjątkiem grupy P_CaL_B. Owariektomia obniżyła stężenie PTH w surowicy, podczas gdy wszystkie zmodyfikowane diety go zwiększyły. Dodatek alendronianu zmniejszył stężenie OC w porównaniu z grupą kontrolną.

W pracy **„Calcium carbonate-enriched pumpkin affects calcium status in ovariectomized rats"** zaobserwowano, że w grupie P_CaC_B nastąpił znaczny wzrost

stężenia PINP w surowicy w porównaniu z grupą kontrolną. W grupach P_CaC i CaC_B stężenie PTH wzrosło w porównaniu z grupą OVX_C. Grupa P_CaC, CaC_B i P_CaC_B wykazały wzrost stężenie wapnia w kości udowej. Stosowanie diet zmodyfikowanych doprowadziło do znacznego wzrostu zawartości wapnia w nerkach (prawie dwukrotnie w grupie P_CaC, trzykrotnie w grupie CaC_B i pięciokrotnie w grupie P_CaC_B). W grupach CaC_B i P_CaC_B liczba osteoblastów i osteocytów wzrosła w porównaniu z grupą OVX_C. Ponadto owariektomia zmniejszyła liczbę osteoklastów i zwiększyła zawartość tłuszczu w szpiku kostnym, a modyfikacje diet nie zmieniły tego wpływu. Owariektomia również spowodowała wzrost procentowej zawartości kości woven, czemu zapobiegał dodatek alendronianu i wzbogaconej dyni bez leku.

W pracy „**Effects of ovariectomy and calcium enriched pumpkin on magnesium status in rats”** wykazano, że owariektomia istotnie obniżyła zawartość magnezu w kościach, podczas gdy w grupach DEF, CaL, CaL_B, P_CaL czy P_CaL_B nastąpiło zwiększenie magnezu w kościach w porównaniu z grupami C i OVX_C. Owariektomia doprowadziła również do zmniejszenia zawartości magnezu w sierści, jednak interwencja dietetyczna nie spowodowała żadnych zmian w sierści, z wyjątkiem grupy CaL_B. Grupa CaL, P_CaL zwiększyły zawartość magnezu w wątrobie w porównaniu z grupą C i OVX_C, a alendronian nasilił ten efekt. Natomiast w nerkach alendronian istotnie zwiększył zawartość magnezu w grupach CaL_B i P_CaL_B, w porównaniu z grupami CaL i P_CaL. Owariektomia zwiększyła zawartość magnezu w mięśniach w porównaniu z grupą kontrolną, na którą interwencja żywieniowa nie miała wpływu. Grupa P_CaL wykazała wyższą zawartość magnezu w wątrobie i mięśniach, w porównaniu z grupą CaL. Połączenie dyni i alendronianu zwiększyło zawartość magnezu w kościach i wątrobie w porównaniu z grupą CaL.

W artykule „**Effect of pumpkin enriched with calcium lactate on iron status in an animal model of postmenopausal osteoporosis”** stwierdzono, że owariektomia nie wpłynęła na stężenie hemoglobiny (HGB) we krwi pełnej, natomiast w grupie P_CaL_B nastąpił istotny wzrost stężenia HGB w porównaniu z grupą kontrolną. Owariektomia znacząco obniżyła zawartość żelaza w kościach, sierści i nerkach w porównaniu z grupą kontrolną, podczas gdy zmodyfikowane diety nie spowodowały żadnych zmian. Owariektomia znacznie obniżyła stężenie żelaza w śledzionie i wątrobie u szczurów, podczas gdy wzbogacona dynia odwróciła ten efekt, który został wzmocniony przez dodanie alendronianu (P_CaL i P_CaL_B). Owariektomia nie wpłynęła na zawartość

żelaza w mięśniach, natomiast stężenie żelaza w mięśniach zostało obniżone u szczurów karmionych wzbogaconą dynią w porównaniu z grupami C i OVX_C.

Komentarz: Dodatek do diety dyni wzbogaconej w sole wapnia powodował wzrost wapnia w kości udowej szczurów. Prawdopodobnym powodem zwiększonej zawartości wapnia w kości po spożyciu wzbogaconej dyni jest zwiększone wchłanianie wapnia przez składniki zawarte w dyni, tj. inulina, karotenoidy, zeaksantyna, luteina, które mogą dodatkowo zapobiegać resorpcji kości u szczurów po owariektomii (Ozaki i in., 2015; Takeda i in., 2017; Tominari i in., 2017; Yamaguchi, 2012). Duży napływ jonów do organizmu jest następnie rozprowadzany do tkanek zawierających receptory wapniowe (CaSR). Poprzez te receptory jony wapnia ze środowiska zewnętrznego przedostają się do środowiska wewnętrznego, zwiększając tym samym zawartość wapnia w tkankach (Bronner, 2001; Cashman, 2002). Wzbogacona dynia w podobny sposób zwiększa zawartość wapnia w kościach co alendronian (dynia wzbogacona w węglan wapnia jest bardziej efektywna niż wzbogacona w mleczan wapnia). To cenne odkrycie, ponieważ wskazuje, że połączenie wapnia z dynią może zapobiegać resorpcji kości i przyczyniać się do wzrostu gęstości kości.

W badaniach zaobserwowano istotny wzrost stężenia wapnia w nerkach szczurów spożywających wzbogaconą dynię oraz alendronian. Znacznie zwiększone stężenie wapnia w nerkach po spożyciu wzbogaconej dyni było nieoczekiwane i mogło prowadzić do poważnych komplikacji w organizmie. Efekt ten zaobserwowano zarówno po zastosowaniu dyni wzbogaconej w węglan jak i mleczan wapnia. Oprócz wapnia, wzbogacona dynia zawiera też inne składniki, które mogą wpływać na czynność nerek, np. witaminy A i E (Kulczynski, Gramza-Michałowska, 2019). Nadmierna ilość tych witamin może prowadzić do hiperfiltracji kłębuszkowej i hiperkalcemii (Parente Filho i in., 2020). Biologicznie aktywną formą witaminy A jest kwas retinowy, który może prowadzić do progresji choroby kłębuszków nerkowych (Chen i in., 2021; Kedishvili, 2016). W badaniach innych autorów dynia ma raczej pozytywny wpływ na nerki (Makni i in., 2010; Medjakovic i in., 2016; Oyetayo i in., 2020). Natomiast silne powinowactwo alendronianu do jonów wapnia powoduje powstawanie agregatów i kompleksów (Papapetrou, 2009), które mogą zatrzymywać się w nerkach, uszkadzając kanaliki nerkowe i powodując ich martwicę (Perazella, Markowitz, 2008). Możemy jedynie założyć, że w nerkach szczurów otrzymujących wzbogaconą dynię lub alendronian wystąpiły niekorzystne zmiany (takie jak kamienie nerkowe i zwapnienie). Obecnie kontynuujemy

badania nad analizą nerek w celu zbadania przyczyny odkrytego przez nas zjawiska. Wydaje się, że dynia wzbogacona w wapń, zwłaszcza w połączeniu z alendronianem, może zwiększać ryzyko dysfunkcji nerek u kobiet w okresie menopauzy.

Podawanie szczurom wzbogaconej dyni skutkowało również zmianami stężeń hormonów związanych z metabolizmem wapnia. Dynia wzbogacona w mleczan wapnia powodowała efekt podobny do podania ES (obniżenie stężenia PINP, czyli hamowanie obrotu kostnego), z wyjątkiem grupy P_CaL_B. Odwrotny efekt na PINP widoczny w P_CaL_B może wynikać z interakcji między alendronianem a wzbogaconą dynią. Zjawisko to wymaga dalszych badań. Ponadto zaobserwowano, że zarówno dynia wzbogacona w mleczan, jak i węglan wapnia, normalizowała poziom PTH u szczurów po owariektomii. Wiadomym jest, że stężenie PTH zależy od ilości wapnia we krwi. Jednak nasze wyniki tego nie potwierdziły. Zmodyfikowane diety mogły mieć wpływ na inne czynniki biochemiczne lub hormonalne związane z parametrami eksperymentalnymi. Ponadto zaskakujące było znalezienie dodatniej korelacji między stężeniem wapnia w kości udowej po podaniu dyni wzbogaconej w węglan wapnia a stężeniem PTH. Wydaje się, że w grupach z dynią i alendronianem obserwowane zależności są związane z dużą kumulacją wapnia w nerkach. PTH stymuluje reabsorpcję wapnia w nerkach i sprzyja jego gromadzeniu. Zaobserwowane zależności mają niewątpliwie aspekt wielokierunkowy.

Zaobserwowano też, że owariektomia spowodowała znaczny wzrost procentowej zawartości kości woven, podczas gdy dodanie do diety dyni wzbogaconej w węglan wapnia i alendronianu przyniosło efekt odwrotny. Można zatem wnioskować, że obniżenie poziomu ES doprowadziło do konieczności odbudowy kości u szczurów po owariektomii. Natomiast wzbogacona dynia i alendronian przyspieszyły odbudowę kości poprzez zwiększenie tworzenia kości, zmniejszając udział kości woven. Odnotowano także dużą liczbę osteoblastów i osteocytów u szczurów po owariektomii; jednak wzrost liczby tych komórek był statystycznie istotny tylko w grupach, które otrzymywały dietę z alendronianem, co sugeruje, stymulację różnicowania osteoblastów, a tym samym intensyfikowanie procesów budowy kości (Ma i in., 2018).

Po owariektomii i podaniu dyni wzbogaconej w mleczan wapnia oraz diety z alendronianem wystąpiły również zmiany w gospodarce magnezu i żelaza. Zawartość magnezu we włosach i surowicy jest ściśle związana z BMD (C. H. Song i in., 2007), dlatego w grupie OVX_C zaobserwowano spadek zawartości magnezu zarówno

w kościach, jak i w sierści szczurów. Odnotowano również zwiększenie zawartości magnezu w kościach udowych u szczurów spożywających dietę deficytową w wapń (grupa DEF). Magnez jest niezbędny do prawidłowego budowania macierzy kostnej poprzez stymulację produkcji blaszek kostnych, mineralizację i odpowiednią aktywność osteoblastów (Rude, Gruber, 2004). Brak prawidłowej podaży wapnia sprawia, że jony magnezu ulegają zwiększonemu odkładaniu na powierzchni hydroksyapatytu (Castiglioni i in., 2013).

Nagromadzenie magnezu w wątrobie i mięśniach można wiązać z obecnością inuliny w diecie szczurów otrzymujących dynię, która jest jednym z czynników zwiększających wchłanianie magnezu (Coudray i in., 2003; Schuchardt, Hahn, 2017). Inulina i nietrawione oligosacharydy wpływają zarówno na metabolizm wapnia, jak i magnezu, zwiększając aktywny i pasywny transport tych składników mineralnych (Scholz-Ahrens, Schrezenmeir, 2002), co może zwiększać zawartość magnezu w tkankach. W niniejszym badaniu zaobserwowano znaczny wzrost zawartości magnezu w nerkach po podaniu alendronianu. Prawdopodobnie poprzez gromadzenie się wapnia w nerkach w wyniku podawania tego leku zostaje zaburzone odpowiednie usuwanie magnezu z organizmu (Gröber, 2019).

Obserwowana w tym badaniu obniżona zawartość żelaza w kościach, sierści, śledzionie, wątrobie i nerkach u szczurów po owariektomii może być spowodowana upośledzoną absorpcją żelaza w dwunastnicy oraz upośledzonym transportem żelaza do krwi (Pagani i in., 2019). Zaobserwowano, że dynia wzbogacona w mleczan wapnia zwiększała zawartość żelaza w tkankach szczurów po owariektomii. Istnieją dowody na to, że inulina zmniejsza wykorzystanie żelaza przez bakterie jelitowe, a tym samym zwiększa wchłanianie żelaza (Costa i in., 2020; Patterson i in., 2009). Ponadto inulina zwiększa powierzchnię chłonną i stymuluje powstawanie w jelitach krótkołańcuchowych kwasów tłuszczowych (SCFA) (Scholz-Ahrens, Schrezenmeir, 2007), które przyczyniają się do wzmocnienia powierzchni chłonnej poprzez namnażanie się komórek nabłonka (Salovaara i in., 2003). SCFA obniżają również pH, promując kwaśne środowisko, które sprzyja konwersji Fe^{3+} do Fe^{2+}, co zwiększa absorpcję żelaza (Yilmaz, Li, 2018). Zwiększona produkcja SCFA będąca efektem działania inuliny, prowadzi do aktywacji białka AMP (adenozynomonofosforan) w mięśniach (Yamashita i in., 2009). Natomiast aktywowana przez AMP kinaza białek mięśniowych bierze udział w hamowaniu żelazo-zależnej apoptozy komórek (ferroptozy) wywołanej stresem oksydacyjnym (Lee i in., 2020). Związek między AMP a żelazem może częściowo wyjaśniać najniższe stężenie żelaza

obserwowane w mięśniach w grupie P_CaL. Badanie wykazało, że dynia wzbogacona w mleczan wapnia i inulinę zwiększa zawartość żelaza w tkankach miękkich, natomiast nie ma wpływu na stężenie żelaza w kościach, co jest zgodne z badaniami innych autorów (Jolliff, Mahan, 2012). Wykazano, że dynia wzbogacona w mleczan wapnia zwiększa zawartość żelaza w wątrobie i śledzionie oraz stężenie HGB u szczurów po owariektomii. Wykazane u szczurów działanie wzbogaconej dyni na gospodarkę żelaza wydaje się mieć pozytywny efekt, jednak u kobiet po menopauzie te wyniki mogą być niekorzystne. Po menopauzie bowiem poziom ferrytyny i żelaza zwykle wzrasta, więc dynia wzbogacona w wapń może powodować gromadzenie się żelaza w organizmie i przyspieszać utratę masy kostnej.

Nowatorskość badań

Nowatorski charakter badania wynika przede wszystkim z zastosowania innowacyjnego produktu spożywczego jakim jest dynia wzbogacona w wapń. Po raz pierwszy zastosowano dynię wzbogaconą w mleczan, jak i węglan wapnia w eksperymencie z udziałem szczurów po owariektomii. Taki rodzaj żywności funkcjonalnej nie był dotychczas zastosowany w żadnym badaniu dotyczącym osteoporozy i metabolizmu wapnia, magnezu i żelaza. Ponadto, w celu oceny zdrowia kości zastosowano szczegółowe badana histopatologiczne oraz biochemiczne. Porównano również działanie wzbogaconej dyni z lekiem powszechnie stosowanym u kobiet z osteoporozą pomenopauzalną – alendronianem. Ponadto w niniejszej pracy działanie dyni wzbogaconej w wapń porównano też z działaniem soli wapnia stosowanymi oddzielnie. Badano interakcje wapnia z innymi pierwiastkami, co daje szerokie spojrzenie na korzystne i ewentualnie niekorzystne skutki działania wzbogaconej dyni, a to stanowi dobrą podstawę do rozpoczęcia ewentualnych badan klinicznych.

Dodatkowo w artykułach nienależących do cyklu pracy doktorskiej wykazano redukcyjny wpływ dyni wzbogaconej w mleczan wapnia na masę ciała szczurów po owariektomii, a także znacznie niższe stężenie leptyny w surowicy (Wawrzyniak, Suliburska i in., 2021). Dynia wzbogacona w mleczan wapnia sprzyja też obniżeniu stężenia aminotransferazy alaninowej i triacylogliceroli we krwi (Wawrzyniak, Gramza-Michałowska i in., 2021). Natomiast dodatek mleczanu wapnia do diety wykazuje

działanie obniżające stężenie cyklooksygenazy I w surowicy u szczurów po owariektomii (Wawrzyniak i in., 2022).

W przygotowaniu są dalsze publikacje naukowe, które obejmują wyniki niniejszego projektu.

Ograniczenia badań własnych

Badanie to miało szereg ograniczeń, które mogły wpłynąć na wyniki. Eksperyment nie obejmował grupy pozorowanej (Sham) - porównaliśmy jednak efekt usunięcia jajników z nieoperowaną grupą kontrolną. Ponieważ w badaniu nie pobierano moczu szczurów, nie mogliśmy stwierdzić, czy wydalanie wapnia wzrastało wraz z gromadzeniem tego pierwiastka w nerkach. Inne parametry związane z metabolizmem kostnym, takie jak stężenie witaminy K, magnezu, żelaza, nie były analizowane w surowicy, ponieważ od szczurów uzyskano jedynie ograniczoną objętość krwi. Nie analizowano również metabolizmu witaminy D i fosforu, które są związane z metabolizmem wapnia i kości. Ponadto analizowano tylko wybrane parametry metabolizmu żelaza; na przykład nie analizowano poziomu ferrytyny i hepcydyny we krwi. Badanie nie obejmowało również analizy histologicznej nerek, co mogłoby być pomocne w wyjaśnieniu mechanizmu gromadzenia wapnia w tej tkance.

Wnioski

1. Dynia wzbogacona w mleczan lub węglan wapnia zwiększa stężenie wapnia w kości udowej oraz poprawia metabolizm kości u szczurów po owariektomii.
2. Dynia wzbogacona w wapń powoduje akumulację wapnia w nerkach szczurów po owariektomii.
3. Alendronian w połączeniu ze wzbogaconą dynią sprzyja akumulacji wapnia i magnezu w nerkach szczurów po owariektomii.
4. Dynia wzbogacona w wapń wpływa na stężenie magnezu i żelaza w kościach oraz tkankach szczurów po owariektomii.
5. Owariektomia powoduje zmniejszenie zawartości wapnia, magnezu i żelaza w tkankach szczurów.

Wniosek praktyczny: Zastosowanie dyni wzbogaconej w wapń może działać korzystnie na gospodarkę wapnia i zdrowie kości u kobiet po menopauzie. Jednak efektem ubocznym tego innowacyjnego produktu, szczególnie w połączeniu z alendronianem może być gromadzenie się wapnia w nerkach. Mechanizm tego szkodliwego działania należy wyjaśnić w dalszych badaniach.

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Streszczenie w języku polskim

Wstęp: Osteoporoza to choroba charakteryzująca się stopniowym ubytkiem masy kostnej, co prowadzi do osłabienia struktury kości i zwiększenia ryzyka złamań. Osteoporoza pomenopauzalna występuje zwykle ok. 45-55 roku życia w wyniku zmniejszenia się ilości hormonów żeńskich, zwłaszcza estrogenu. Wapń jest głównym składnikiem mineralnym kości, dlatego odpowiednia jego podaż jest ważnym czynnikiem w prewencji i terapii osteoporozy. Skutecznym i łatwym sposobem na zwiększenie ilości wapnia w diecie jest zastosowanie żywności wzbogaconej w wapń. Jednym z takich innowacyjnych produktów jest dynia wzbogacona w wapń poprzez odwodnienie osmotyczne przy użyciu inuliny.

Celem pracy było określenie wpływu wybranych czynników żywieniowych i farmakologicznych na gospodarkę mineralną i metabolizm tkanki kostnej w zwierzęcym modelu osteoporozy pomenopauzalnej.

Materiały i metody: Sto 12-miesięcznych szczurów typu Wistar zostało podzielonych na 10 grup. 90 szczurom usunięto jajniki (OVX), aby stworzyć szczurzy model osteoporozy pomenopauzalnej. Następnie przeprowadzono 12-tygodniową interwencję żywieniową: grupa kontrolna (C) i jedna z grup z wyciętymi jajnikami (OVX_C) otrzymywały standardową dietę (bez modyfikacji), grupa DEF otrzymywała dietę z deficytem wapnia, grupa CaC_B otrzymywała dietę standardową z dodatkiem alendronianu, grupa P_CaC dostawała dietę z dynią wzbogaconą w węglan wapnia, grupa P_CaC_B otrzymywała dietę z alendronianem i dynią wzbogaconą w węglan wapnia, grupa CaL była karmiona standardową dietą z mleczanem wapnia, grupa P_CaL była karmiona dynią wzbogaconą w mleczan wapnia, grupie CaL_B podawano alendronian i mleczan wapnia, a grupie P_CaL_B - alendronian i dynię wzbogaconą mleczanem wapnia. Dynia została wcześniej wzbogacona solami wapnia w procesie odwadniania osmotycznego przy pomocy inuliny.

Po zakończeniu doświadczenia szczury dekapitowano i pobierano krew oraz tkanki. W surowicy oznaczono stężenie N-końcowego propeptydu prokolagenu typu I (PINP), parathormonu (PTH), estrogenu i osteokalcyny (OC) za pomocą testu immunoenzymatycznego Elisa. Przeprowadzono analizę morfologiczną krwi pełnej. Stężenie wapnia w surowicy oraz zawartość wapnia, magnezu i żelaza w tkankach zostały określone metodą spektrofotometrii atomowo-absorpcyjnej.

Wyniki: Zaobserwowano, że owariektomia spowodowała spadek zawartości wapnia, magnezu i żelaza w tkankach szczurów. Wykazano też, że w grupach P_CaC i P_CaL zwiększona została zawartość wapnia w kościach udowych szczurów po owariektomii. W grupach CaC_B i P_CaC_B liczba osteoblastów i osteocytów wzrosła w porównaniu z grupą OVX_C. Dodatkowo zaobserwowano, że w grupach P_CaC i CaC_B zmniejszony został procentowy udział kości woven w porównaniu z grupą OVX_C. Zaobserwowano też, że diety modyfikowane obniżyły stężenie PINP w surowicy, z wyjątkiem grupy P_CaL_B i P_CaC_B, natomiast zwiększyły stężenie PTH w surowicy. Dodatek alendronianu zmniejszył stężenie OC w porównaniu z grupą kontrolną.

W grupach P_CaC i P_CaL nastąpiła akumulacja wapnia w nerkach, a efekt ten został spotęgowany dodatkiem alendronianu. Również stężenie magnezu w nerkach zostało zwiększone w grupach otrzymujących lek. Dieta DEF zwiększyła zawartość magnezu w kościach w porównaniu z grupą OVX_C. Zaobserwowano również, że w grupie P_CaL_B nastąpił istotny wzrost stężenia hemoglobiny w porównaniu z grupą kontrolną.

Wnioski: Dynia wzbogacona w mleczan lub węglan wapnia zwiększa stężenie wapnia w kości udowej oraz poprawia metabolizm kości u szczurów po owariektomii. Dynia wzbogacona w wapń powoduje również akumulację wapnia w nerkach szczurów po owariektomii, a alendronian w połączeniu ze wzbogaconą dynią sprzyja akumulacji wapnia i magnezu w nerkach szczurów po owariektomii. Natomiast owariektomia powoduje zmniejszenie zawartości wapnia, magnezu i żelaza w tkankach szczurów.

Streszczenie w języku angielskim

Introduction: Osteoporosis is a disease characterized by a gradual loss of bone mass, which leads to weakening of the bone structure and an increased risk of fractures. Postmenopausal osteoporosis usually occurs around the age of 45-55 as a result of a decrease in the amount of female hormones, especially estrogen. Calcium is the main mineral component of bones, therefore its adequate supply is an important factor in the prevention and treatment of osteoporosis. An effective and easy way to increase the amount of calcium in a diet is to use calcium-enriched foods. One of such innovative products is pumpkin enriched with calcium through osmotic dehydration using inulin.

The aim of the study was to determine the effect of selected nutritional and pharmacological factors on mineral metabolism and bone tissue metabolism in an animal model of postmenopausal osteoporosis.

Materials and methods: One hundred 12-month-old Wistar rats were divided into 10 groups. 90 rats had their ovaries removed (OVX) to create a rat model of postmenopausal osteoporosis. Then, a 12-week nutritional intervention was performed: the control group (C) and one of the ovariectomized groups (OVX_C) received a standard diet (without modification), the DEF group received a calcium-deficient diet, the CaC_B group received a standard diet with alendronate, the P_CaC group were fed a diet with calcium carbonate-enriched pumpkin, the P_CaC_B group were fed a diet with alendronate and calcium carbonate-enriched pumpkin, the CaL group were fed a standard calcium lactate diet, the P_CaL group was fed calcium lactate-enriched pumpkin, the CaL_B group were fed alendronate and calcium lactate, and the group P_CaL_B - alendronate and pumpkin enriched with calcium lactate. The pumpkin was previously enriched with calcium salts in the process of osmotic dehydration with inulin.

At the end of the experiment, the rats were decapitated and blood and tissues were collected. Serum concentrations of procollagen type I N propeptide (PINP), parathyroid hormone (PTH), estrogen and osteocalcin (OC) were determined using the enzyme-linked immunosorbent assay (ELISA). Complete blood count analysis was performed. Serum calcium and tissue calcium, magnesium and iron content were determined by atomic absorption spectrophotometry.

Results: It was observed that ovariectomy caused a decrease in the content of calcium, magnesium and iron in rat tissues. It was also shown that in groups P_CaC and P_CaL the content of calcium in the femur bones of rats after ovariectomy was increased.

In the CaC_B and P_CaC_B groups, the number of osteoblasts and osteocytes increased compared to the OVX_C group. In addition, it was observed that in the P_CaC and CaC_B groups, the percentage share of woven bones was reduced compared to the OVX_C group. It was also observed that the modified diets decreased the serum PINP concentration, except for the P_CaL_B and P_CaC_B groups, and increased the serum PTH concentration. The addition of alendronate decreased the concentration of OC compared to the control group.

In the P_CaC and P_CaL groups, there was an accumulation of calcium in the kidneys, and this effect was enhanced by the addition of alendronate. Also, the concentration of magnesium in the kidneys was increased in the groups receiving the drug. The DEF diet increased bone magnesium content compared to the OVX_C group. It was also observed that in the P_CaL_B group there was a significant increase in hemoglobin concentration compared to the control group.

Conclusions: Pumpkin enriched with calcium lactate or calcium carbonate increases the calcium concentration in the femur and improves bone metabolism in ovariectomized rats. Calcium-enriched pumpkin also causes calcium accumulation in the kidneys of ovariectomized rats, and alendronate in combination with enriched pumpkin promotes calcium and magnesium accumulation in the kidneys of ovariectomized rats. On the other hand, ovariectomy reduces the content of calcium, magnesium and iron in rat tissues.

Artykuły naukowe stanowiące cykl publikacji

Lead Article

Nutritional and health factors affecting the bioavailability of calcium: a narrative review

Natalia Wawrzyniak and Joanna Suliburska

Calcium is responsible for the effectiveness of various processes, and its supply in the diet is necessary for the normal function of the human body. Apart from being an important component of the skeleton, calcium also helps maintain the structure of cell organelles and regulates intracellular and extracellular fluid homeostasis. This review presents the nutritional and health factors that affect the bioavailability of calcium. Physiological conditions and factors such as pregnancy, infancy, menopause, old age, hormones, growth factors associated with calcium metabolism, diseases limiting its absorption, and intestinal microbiota are distinguished among endogenous factors. Although the calcium supply in the body is genetically conditioned and specific to each person, its qualitative and quantitative composition can be modified by external factors. The exogenous factors include dietary modifications with particular nutrients and pharmacological treatment. Adequate calcium levels increase bone protection and prevent osteoporosis, a disease involving low mineral bone mass.

INTRODUCTION

On average, there is > 1 kg of calcium in each adult human body. Essentially all (99%) of this calcium content can be found in bones in the form of hydroxyapatite, which forms an inorganic matrix with phosphate. The remaining 1% of calcium circulates in the blood as free calcium, ionized with plasma proteins or associated with anions, such as citrate or lactate. Calcium must be supplied with food to meet this demand. Calcium is lost through urination, evacuation, sweating, hair loss, and exfoliation.[1]

Calcium ions are essential for living organisms and for normal body function. It is involved in muscle contraction, cell death, transmission of nerve impulses, cell differentiation, enzyme activation, and immune response. Disorders of calcium status contribute to the development of bone diseases and increase the risk of epithelial cancer and metabolic diseases. Providing the right amount of calcium in the diet is thus necessary for normal body function.[2]

The diet of the population deficient in calcium represents a problem globally. Few countries can boast a sufficient average intake of this essential mineral. The majority of the world's population consumes < 1000 mg calcium daily. The lowest average consumption is seen in east, south, and southeast Asia (< 400–500 mg/day), with Nepal coming last with an average consumption of 175 mg/day. Average calcium consumption levels of 400–700 mg/day have been recorded in Africa and South America, whereas > 1000 mg/day is consumed in the countries of northern Europe, with the highest average supply in Iceland, at 1233 mg/day.[3]

There are 2 pathways through which calcium can be absorbed from the gut lumen into the blood: transcellular and paracellular. Transcellular calcium

Affiliation: *N. Wawrzyniak* and *J. Suliburska* are with the Department of Human Nutrition and Dietetics, Faculty of Food and Nutrition Science, Poznań University of Life Sciences, Poznań, Poland.

Correspondence: *J. Suliburska*, 31 Wojska Polskiego St, 60-624 Poznań, Poland. E-mail: joanna.suliburska@up.poznan.pl.

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absorption is an active process of saturation against a concentration gradient and is the dominant means of calcium absorption in the initial segments of the intestine (ie, the duodenum and jejunum). Epithelial calcium ion channels participate in this path, including calcium-selective transient receptor potential vanilloid (TRPV) subfamily member 6 (TRPV6), TRPV subfamily member 5 (TRPV5), voltage-dependent L-type α1 D subunit calcium channels, calbindin proteins (CB; mainly CB9k), and sodium ion and calcium ion (Na^+/$Ca2^+$) exchanger and plasma membrane calcium pumps (PMCAs; mainly PMCA1b). Paracellular calcium absorption usually takes place in the ileum and jejunum, where calcium ions are absorbed passively with the gradient by tight junctions—intercellular structures located in places where the plasma membranes of adjacent cells are very close. The transmembrane proteins belonging to tight junction structures include claudins and occludin. Paracellular transport predominates when the calcium ion inflow to the intestines is sufficient or excessive.[2]

Total serum calcium concentration should range from 8.8 mg to 10.4 mg/dL, and the ionized (free) calcium concentration should be 4.6–5.3 mg/dL in healthy people. When the ionized calcium level drops below 3 mg/dL, hypocalcemia may develop, manifested by tingling fingers, muscular and nervous sensitivity, or perioral paresthesia. Dietary calcium deficiency can also disrupt bone metabolism, causing osteopenia or osteoporosis.[1] Low calcium supply may lead to increased blood pressure.[4] However, when serum calcium levels rise above the norm (eg, due to inadequate supplementation), hypercalcemia can develop, which causes symptoms such as apathy, malaise, and an inability to concentrate.[1] The formation of kidney stones in response to calcium supplementation is a controversial topic. A daily intake of $\geq$ 1000 mg of calcium not only does not cause the formation of stones but also protects against this disorder. Calcium binds to the oxalates in the gut, which are components of kidney stones, and reduces their absorption.[4]

The proportion of bone components is variable, with the composition changing continuously. There are 2 main directions of remodeling: bone formation (the anabolic process) and bone resorption (the catabolic process). Bone turnover is a physiological phenomenon causing old bone tissue to be replaced by new tissue. During these changes, substances called bone turnover markers enter the blood and urine. Measurements of their concentration can help identify bone metabolic activity. Bone markers include C-terminal propeptides of type I collagen, N-terminal propeptides of type I collagen, and bone-specific alkaline phosphatase and osteocalcin, levels of which reflect osteoblast (ie, osteogenic cell) activity. The bone resorption markers include tartrate-resistant acid phosphatase 5 b, pyridinolines, N-terminal telo-peptide of type I collagen, and C-terminal telopeptide of type I collagen, levels of which reflect the activity of osteoclasts (osteoclastic cells).[5] The main regulator of bone remodeling is the RANKL/RANK/OPG system, which optimizes osteoclast formation, thus preventing excessive bone resorption. Unbalanced bone changes manifested by abnormal concentrations of bone turnover markers may be evidence of postmenopausal osteoporosis.[6]

There needs to be a balance between calcium absorption in the intestines, calcium incorporation, bone resorption, renal calcium excretion, and reabsorption for calcium phosphate homeostasis in the body. Calcium metabolism is regulated by 3 hormones: parathyroid hormone (PTH), calcitonin, and vitamin D_3. Parathyroid hormone affects calcium reabsorption in the kidneys, phosphorus excretion, mobilization of phosphate and calcium from bones, and hydroxylation of calcidiol (25-cholecalciferol) into the active form of vitamin D_3 (calcitriol). Calcitonin stimulates the excretion of calcium by the kidneys and inhibits osteoclast activity. Calcium release from bone thus is inhibited and its level in the blood is reduced. Vitamin D_3 increases enteral calcium absorption and its reabsorption in the kidneys (similar to PTH). Calcium homeostasis is also controlled by estrogens, corticoids, and thyroxine. Estrogens not only increase calcium absorption in the intestines and its reabsorption in the kidneys but also protect bones from resorption. Corticoids promote calcium excretion and reduce its enteral absorption, thus reducing calcium levels in the body.[7] Although thyroxine increases calcium absorption in bones, calcium absorption may decrease with hyperthyroidism (ie, thyroxine level over the normal limit), due to accelerated absorption of calcium ions from the intestinal lumen into the blood.[8]

Calcium is mainly eliminated from the body through the kidneys. Calcium removal is necessary to maintain homeostasis and to prevent excessive deposition of calcium crystals in tissues and fluids. Apart from the excretion of calcium, the kidneys are also responsible for its reabsorption. Approximately 60%–70% of calcium that has been filtered through the glomeruli is reabsorbed. As in the intestine, there are 2 pathways of calcium transport in the kidneys. The paracellular pathway, which is dominant in calcium reabsorption, involves connecting proteins called claudins, which are selective for barrier or pore formation as well as for the passage of cations and anions. The molecular structures involved in the transcellular pathway have not yet been described in detail.[9] The kidneys indirectly affect the bioavailability of calcium by participating in the

hydroxylation of calcidiol to the active form of vitamin D_3.[10] In this narrative review, we describe the most recent studies (published in 2015–2020) of the nutritional and health factors that affect the bioavailability of calcium in humans.

ENDOGENOUS FACTORS

Ontogenesis

Pregnancy, childbirth, and breastfeeding. Calcium absorption doubles from the first trimester of pregnancy, partly due to an increase in TRPV5/6, CB9k, PMCA1, Na^+/Ca^{2+} exchanger, claudin-2 and claudin-12, and partly due to induction by calcitriol. These changes occur to meet the fetus' demand for calcium. However, after childbirth, there is increased bone resorption due to changes in metabolism. This condition is independent of the diet, duration of breastfeeding, and restoration of menstruation.[11] There is also evidence that bone loss is associated with increased release of lead from the skeleton. Slowing the mother's bone resorption might perhaps not only help maintain adequate bone density but also limit the negative heavy-metal load of the fetus and breastfed infant.[12] During pregnancy and lactation, an adequate calcium intake is necessary due to the rapid growth and development of the skeleton of the child in the womb and in the first stage of life after birth. When it is difficult to ensure an adequate calcium supply from food products, supplementation is necessary to prevent resorption of the pregnant woman's bones.[13] Breastfeeding is beneficial for the child's development. Breastfeeding for at least 6 months protects the woman from osteoporosis, because endocrine changes occurring in the body cause increased calcium absorption in the intestines and its reabsorption in the kidneys.[14] Prolactin, a milk-producing hormone, is also responsible for increasing calcium absorption during pregnancy and lactation. Prolactin stimulates transcellular and also paracellular calcium absorption by the intestinal epithelium. Prolactin adapts the intestinal mucosa by increasing the height of the small intestinal villi and deepening the crypts. This transformation increases the absorbent surface of the intestinal mucosa. Prolactin also increases the expression of the calcium transporters PMCA1b and TRPV6.[15] When prolactin is secreted, calcium absorption is independent of calcitriol. However, after the lactation period, calcitriol affects transcellular calcium transport again. Kidneys play an important role in positive calcium balance. Peak calcium reabsorption occurs during breastfeeding and decreases after maturation.[16] Because calcium absorption is disturbed in premature infants, feeding with human milk-fortifier seems beneficial. Human-milk fortifier can increase calcium absorption, ensuring the same increase as in children fed artificial milk, while reducing the risk of food intolerance and sepsis, and preventing necrotic enteritis.[17]

Menopause. Until approximately the third decade of life, anabolic reactions predominate over catabolic ones. After this period, bone mineral density decreases at different rates depending on various factors. The bone density of perimenopausal women is much lower than that of women 30 years old. Both estrogens and androgens protect the mammalian skeleton, strengthen bones, and affect their growth. A sharp drop in estrogen levels due to the cessation of ovarian function significantly deteriorates the bone structure. Within 3 years after the last menstruation, bone strength and mass decrease. A bone density below −2.5 SD indicates postmenopausal osteoporosis, which is a risk factor for bone fractures.[18–20] As a consequence of increased bone resorption, the level of skeletal components in the blood increases. When calcium is found in the extracellular fluid, it binds to the calcium-sensing receptor in parathyroid cells. The calcium-sensing receptor is involved in calcium metabolism and stimulates the parathyroid glands to stop secreting PTH. As a consequence of low PTH levels, $1,25(OH)_2D$ synthesis in the kidneys decreases and the absorption of calcium in the intestine is reduced.[21,22] After menopause, reduced calcium reabsorption in the kidneys can be observed, caused by the stimulation of calcium-sensing receptor in kidney cells due to the excess of extracellular calcium. Hypercalciuria reference values differ by race: White women excrete more calcium after menopause than Black women. These mechanisms are used to normalize calcium levels in the body striving for homeostasis.[22,23]

Senility. Postmenopausal and senile osteoporosis are classified as primary osteoporosis, meaning that they are not caused by other diseases. Both postmenopausal status and old age lead to large decreases in bone density; thus, not only women but also older men are at risk of osteoporotic fractures. In old age, impaired calcium metabolism results from low hormone levels and from the deterioration of internal organs, especially the kidneys. The kidneys are responsible for the synthesis of vitamin D_3 in the form of calcitriol ($1,25(OH)_2D$). Kidney dysfunction causes a decrease in calcitriol synthesis and less calcium absorption in the intestines. A low calcium level in the body (in severe cases, called hypocalcemia) stimulates the parathyroid glands to secrete PTH, which results in increased calcium mobilization from the bones.[21,24] Parathyroid hormone rapidly inhibits calcium excretion and stimulates the synthesis of vitamin D_3, which increases the absorption of

Table 1 **Regulators of body changes affecting calcium absorption**

Regulator	Effect	Mechanism	References
Estrogen	Increased calcium absorption	Effect on the expression of TRPV6 and PMCA1b proteins in the duodenum	Nie et al (2020)[25] Cleemann et al (2017)[26]
Fibroblast growth factor 23	Limited calcium absorption	Improvement of urinary phosphate excretion and calcidiol catabolism	Rodríguez et al (2015)[27]
		Direct inhibition of calcium absorption of duodenal parenchyma enterocytes	Wongdee et al (2019)[28] Wongdee et al (2016)[15]
PTH	Increased calcium absorption	Increased vitamin D synthesis in the kidneys	Goltzman et al (2018)[21]
IGF-1	Increased calcium absorption	Mediated through the beneficial effects of PTH on bone building	Yakar et al (2018)[29]
		Increased 1α-hydroxylase activity in the proximal tubule of the kidney that converts calcidiol to calcitriol	Van Hemelrijck et al (2015)[30]
		Increased CB9k levels in the duodenum	Areco et al (2015)[8]
		increased calcium absorption despite unchanged blood vitamin D3 levels (direct effect of IGF-1 on calcium absorption)	Matsumoto et al (2018)[31]
Calcitriol (vitamin D)	Increased calcium absorption	Vitamin D receptor and vitamin D stimulate the expression of the calcium channel (TRPV6) in the intestine	Christakos et al. (2017)[32]
		Oral vitamin D analogs (eldecalcitol and α-calcidol) increase fractional calcium absorption in the intestines	Uenishi et al (2018)[33]
		The concentration of calcidiol (the main form of vitamin D in the blood, but with ~100 times lower activity than calcitriol) increases the PTH level in women whose diet is not high in calcium-rich products	Sirichakwal et al (2015)[34]

Abbreviations: IGF-1, insulin-like growth factor-1; PTH, parathyroid hormone.

calcium from the intestinal lumen into the blood.[24] Calcium homeostasis becomes increasingly difficult to maintain in older patients because of the worsening condition of their kidneys and intestines.[21]

Regulators of body changes

Regulators of body changes are another endogenous factor that can stimulate calcium absorption, but some also limit its bioavailability (eg, due to the body's defense against hypercalcemia, excessive calcium levels in the blood). Table 1 lists selected hormones and other regulators that affect the bioavailability of calcium.[8,15,21,25–34]

Estrogen. As mentioned, sex hormones protect the mammalian skeleton. The consequence of a decrease in estrogen levels is bone weakness due to increased mineral resorption and a decrease in calcium absorption in the intestine. Postmenopausal osteoporosis is associated with a decrease in estrogen levels in the body and also a decrease in calcium absorption in the intestines. Estrogen affects the expression of TRPV6 and PMCA1b proteins, which are responsible for the transcellular transport of calcium ions, thereby regulating calcium absorption in the duodenum.[25] In women with Turner syndrome (who have primary ovarian failure) who have been treated with estrogen for 5 years, an increase in bone mineral density and a decrease in bone turnover have been observed.[26]

Fibroblast growth factor 23. Recent reports have pointed to the important role of fibroblast growth factor 23 (FGF-23) in calcium and phosphorus homeostasis. FGF-23 has been called phosphate hormone because of this regulatory function. Fibroblast growth factor 23 is produced by osteoblasts and osteocytes (bone cells) and other body cells (ie, in brain, kidneys, liver, lungs, intestinal enterocytes, and spleen), and its main role is to improve urinary phosphate excretion and catabolism of 1,25(OH)2D. When calcitriol breaks down and its concentration decreases, intestinal calcium absorption is also reduced.[27] Fibroblast growth factor also acts directly on the intestinal epithelium. Because its receptors are found in both the basolateral membrane and the apical membrane of duodenal enterocytes, FGF-23 acts both from the basolateral and apical side. It directly inhibits both paracellular and transcellular calcium absorption in duodenal parenchyma. The factors that increase the release of FGF-23 are 1,25(OH)2D, phosphates, PTH, growth of the intestinal mucosa during pregnancy, and lactation. The concentration of

GFG-23 in the intestine is increased by preventing calcium hyperabsorption.[15,28]

Parathyroid hormone. Parathyroid hormone is closely related to calcium metabolism. Parathyroid hormone increases the renal synthesis of vitamin D, which then stimulates intestinal calcium absorption. When calcium levels increase, the calcium-sensing receptor is activated, which prompts the parathyroid glands to stop secreting PTH. This results in a decrease in vitamin D synthesis in the kidneys and, as a result, a decrease in calcium absorption. This process, based on feedback principles, protects the body against hypocalcemia and hypercalcemia. However, there are situations when the parathyroid gland is inactive and does not fulfil its function, failing to release the correct levels of hormones. This may be due to the removal of the thyroid gland (eg, due to the development of cancer) or to irradiation of the neck (eg, radiotherapy). Parathyroid dysfunction is associated with a decrease in PTH activity, a decrease in blood calcium levels, and phosphorus retention in the body.[35]

Insulin-like growth factor 1. Insulin-like growth factor-1 (IGF-1) is thought to be a mediator in the beneficial effects of PTH in bone building. Activity of IGF-1 induces PTH protection of bones and, along with steroid hormones and PTH, IGF-1 is involved in bone anabolic reactions.[29] Insulin-like growth factor-1 affects calcium metabolism by increasing the activity of 1α-hydroxylase, which, in turn, converts inactive calcidiol into the active form of vitamin D, which is a regulator of serum calcium.[30] Administration of IGF-1 to older rats increased calcium absorption, although vitamin D_3 plasma levels remained unchanged, suggesting a direct effect of IGF-1 on calcium absorption. Insulin-like growth factor-1 interacts with calcium absorption through an increase in transcellular pathway efficiency by increasing CB9k levels in the duodenum.[8] The decrease in IGF-1 occurs not only in the elderly but also in patients with nonalcoholic fatty liver disease and in people with alcoholism who have elevated levels of blood transaminases, whereas elevated albumin, bilirubin, and calcium levels are correlated with high IGF-1 levels.[31]

Vitamin D. Vitamin D is a steroidal organic compound that regulates calcium and phosphate homeostasis. To transform vitamin D obtained from food and skin synthesis into its active form, 2 processes are needed. The first occurs in the liver, where vitamin D is hydroxylated by the CYP2R1 enzyme to 25-hydroxycholecalciferol (calcidiol, 25(OH)D). Calcidiol is the main form of vitamin D in the bloodstream, but it has approximately 100 times lower biological activity than calcitriol. The second process takes place in the kidneys, where the active form of vitamin D is formed by CYP27B1 enzyme hydroxylation, namely 1α, 25-hydroxycholecalciferol (calcitriol, 1,25(OH)2D).[36] To understand the role of vitamin D in calcium absorption, it is necessary to understand vitamin D receptor (VDR) and its role in the action of 1,25(OH)2D. The ability to express genes in many body tissues allows VDR to mediate calcitriol and to participate in the regulation of PTH independently of 1,25(OH)2D.[37] The vitamin D receptor found in intestinal, kidney, parathyroid, and bone cells is involved in the coordination of mineral homeostasis. For calcium to be absorbed from the intestinal lumen on a transcellular pathway, it is necessary to form a calcium channel (TRPV6) whose expression depends on vitamin D receptor and its active form.[32] Intestinal vitamin D–receptor stimulation can occur under the influence of calcitriol but also via oral administration of vitamin D analogs, (ie, eldecalcitol and α-calcidol), which increase intestinal fractional calcium absorption.[33] The concentration of calcidiol, which is much lower than that of calcitriol, also affects its availability in women whose diet is not high in calcium-rich products. In Thai postmenopausal women, it has been observed that serum 25(OH)D concentration increases the level of PTH, which, in turn, increases calcium absorption from the intestines.[34]

Diseases

Hypochlorhydria. The effectiveness of calcium absorption in the intestines depends on it previously having been dissolved in the stomach. A very acidic pH (approximately 1.5) is necessary for this to occur. Calcium salts also dissolve best in an acidic environment, so the most effective absorption of calcium ions occurs in the duodenum, where pH is approximately 6. The pH is higher in distal segments of the intestine, so the absorption of calcium into the blood is weaker. The bone mass of patients with hypochlorhydria (ie, insufficient secretion of gastric acid by the parietal cells) of the stomach is lower due to the reduced absorption of calcium from the intestinal lumen into the blood. Also, gastrectomy and the use of proton pump inhibitors may increase pH, which leads to abnormal calcium bioavailability in the gastrointestinal tract. This effect of hydrochloric acid on bone health was observed in a study of mice with cholecystokinin B–receptor deficiency. The receptor stimulates the parietal cells to secrete hydrochloric acid by binding gastrin. Hypochlorhydria can lead to calcium absorption disorders, which lead, in turn, to secondary hyperparathyroidism. A high level of PTH results in excessive bone resorption. Patients with osteoporosis are often treated with bisphosphonates, which

can cause numerous gastrointestinal problems. Proton pump inhibitor treatment is then often prescribed, which results in a counterproductive effect.[38]

Type 1 diabetes. During long-term hyperglycemia, advanced glycation end products are produced in the body. Microvessels are damaged, which causes neuropathies, nephropathies, and retinopathies. Apart from affecting the organs responsible for the regulation of calcium metabolism, diabetes also reduces the level of calcitriol circulating in the body, the number of vitamin D receptors in the intestine, and the amount of CB9k.[39] A study of adolescent girls showed that type 1 diabetes did not substantially affect the quality of calcium absorption, but its renal excretion was increased. This indicates the body's inability to sufficiently use this mineral for bone formation. This inability leads to abnormal formation of the skeletal structure, which is crucial in adolescence, and may lead to osteoporosis at an older age.[40]

Obesity. Obese people are at a high risk of micronutrient deficiency, including calcium and vitamin D. They have low levels of calcidiol, which, by reducing the secretion of PTH, leads to the disturbance of calcium absorption from the intestines. The decreased concentration of calcidiol in the blood probably is due to the abundance of adipose tissue in which vitamin D can accumulate; vitamin D's release into the bloodstream then occurs when fat tissue is reduced during weight loss.[41] The low bioavailability of calcium might point to negative effects on bone metabolism, but the bone mass of obese people tends to be denser than that of nonobese people.[42] Other authors have observed that obese people have higher bone mineral density (BMD) and greater fracture resistance, due to the increased amount of lean body mass caused by the overgrowth of muscle mass and an increase in bone load. The situation is different in the elderly and in those with comorbidities, whose excess body weight is associated with sarcopenia (ie, loss of muscle mass) and thus deterioration of physical fitness. Sarcopenic obesity is caused by an inflammation that progresses with motor inactivity, which, in turn, leads to an increase in fat mass, thus forming a vicious circle. Excessive body weight disrupts bone metabolism not only through inflammatory but also via hormonal factors. The increase in the number of adipocytes (fat cells) is associated with quantitative changes in the secretion of the adipose tissue hormones leptin, adiponectin, and sex hormones.[43] In obese people, an increase in the level of leptin is observed, which stimulates satiety signals in the hypothalamus and thus affects the regulation of appetite.[44] Leptin has both positive and negative modes of action in bone metabolism; on one hand, it inhibits osteoclastogenesis and increases the proliferation of osteoblasts, which demonstrates the beneficial effect of leptin on bone. On the other hand, its deficiency causes an increase in the thickness and volume of the bone trabecula, confirming the antiosteogenic role of leptin.[42] The profile of bone metabolism in lean people is better, despite the higher leptin levels found in obese people, which can be explained by resistance to leptin and deterioration of cartilage and bone due to the pro-inflammatory status caused by excessive secretion of this hormone.[45] In vivo studies have shown that excess body weight is associated with a decrease in adiponectin levels, which helps improve bone density. Nevertheless, in the perimenopausal period, the BMD of obese women with type 2 diabetes is lower than that of healthy peers, which may be influenced by the presence of comorbidities.[46] Adipocytes can secrete estrogens, but adipose sex hormones have not been observed to significantly affect bone health.[43] The same applies to obese children, who are not uncommon in the urbanized world. Although measurements of bone density in children have been contradictory (mainly due to the use of dual-energy X-ray absorptiometry, which has limitations in measuring the BMD of the developing skeleton), scientists agree on 1 aspect: Obese children are more likely to have bone fractures both during the childhood and later in adult life than their peers with normal body weight. Obesity also makes it impossible to achieve peak body weight at the same level as in children with a normal BMI, which can contribute to the onset of osteoporosis up to 13 years earlier.[47] In both obese adolescents and adults, bone weakness can be exacerbated by a lack of sufficient sun exposure, which reduces the activation of vitamin D in the skin and thus the bioavailability of calcium.[48]

Bariatric surgery. One way to treat advanced obesity is to shrink the stomach. Patients who have undergone Roux-en-Y surgery are at risk of osteoporosis because their bone density decreases. Roux-en-Y gastric bypass is a type of bariatric surgery that involves forming a small bag from the stomach with a bypass of the duodenum and part of the jejunum. As a result, the patient eats smaller portions of meals and is satiated faster, which results in a weight loss. Unfortunately, the absorption surface is reduced due to the omission of the proximal part of the small intestine, which results in limited availability of nutrients and disturbed intestinal calcium absorption. Consequently, the PTH level increases, which leads to increased bone resorption (substantially higher blood C-terminal telopeptide of type I collagen levels).[49]

Coeliac disease. Coeliac disease is an enteropathy, an autoimmune disease manifested by the atrophy of intestinal villi in the presence of gluten. As the absorption surface is reduced, so is the supply of nutrients, which leads to malabsorption and diseases like osteoporosis. The bones of menopausal women with coeliac disease have significantly lower architectural parameters than the bones of healthy peers. A gluten-free diet reduces bone resorption markers and regulates calcidiol and PTH levels. The bone microarchitecture of the patients who fully recovered (with negation of the 3 measured antibodies) improved substantially. A gluten-free diet is necessary for people with coeliac disease to maintain healthy bones.[50]

Chronic pancreatitis. One symptom of chronic pancreatitis is exocrine pancreatic insufficiency, which results in malabsorption of nutrients. The BMD of patients with chronic pancreatitis is much lower than the BMD of healthy people. This is probably caused by deficiencies of vitamin D_3 and calcium. The problem of osteoporosis or ost

eopenia in patients with chronic pancreatitis is usually compounded by smoking, which is an independent risk factor of osteoporosis because of its effect on the hormones involved in bone remodelling.[51]

Microbiota

Intestinal microbiota refers to all the probiotic, commensal, and pathogenic microorganisms inhabiting the intestines. These microorganisms interact with the host, affecting dendritic cells, immune system cells, and hepatocytes. The interaction of the microbiome with the inhabited organism results in products such as indole derivatives, secondary bile acids, polyamines, and short-chain fatty acids (SCFAs). There is evidence that the microbiome and its metabolites may improve the bone structure and reduce bone turnover by increasing calcium bioavailability.[52]

Firmicutes-to-Bacteroidetes ratio. The 2 predominant types of bacteria in the human gut are Firmicutes and Bacteroidetes. External factors, such as antibiotics or food contaminated with heavy metals or pesticides, can alter the microbiota quantitatively and/or qualitatively. The ratio of the number of Firmicutes to Bacteroidetes may have an effect on the state of health and particularly on obesity. Firmicutes bacteria are prevalent in people who eat a Western diet rich in sugar, fat, and protein, whereas people from African regions who eat traditional diets with high fiber content showed a higher proportion of Bacteroidetes. It follows that Firmicutes are more effective at obtaining energy from nutrients than are Bacteroidetes, promoting increased caloric absorption.[53] The altered ratio of Firmicutes to Bacteroidetes may also indicate inflammation caused by lipopolysaccharide, a proinflammatory endotoxin that can pass from the intestine into the blood. When the Firmicutes-to-Bacteroidetes ratio is higher (ie, when there are fewer Bacteriodetes), the body is exposed to chronic lipopolysaccharide exposure.[54] Lipopolysaccharide damages the intestinal epithelium, increasing its permeability and causing inflammation; this process impairs the absorption of minerals, reducing the bioavailability of, for example, calcium and iron.[55] A reduction in the Firmicutes-to-Bacteroidetes ratio leads to an increase in the concentration of calcium ions in the blood and an improvement in the bone microstructure.[56]

Short-chain fatty acids. Short-chain fatty acids, such as butyrate, acetate, and propionate, are formed as a result of microbial fiber fermentation. Their activity results in lower blood pressure, appetite regulation, and glucose homeostasis, improvement of pancreatic function, and strengthening of the intestinal barrier.[39] Insufficient SCFA production leads to increased intestinal permeability and, consequently, to obesity and cardiovascular disease.[57] Short-chain fatty acids can lower the pH of grassy ingredients, thus maintaining the intestinal microecosystem by providing an acidic environment for beneficial bacteria and minimizing the amount of harmful bacteria. A low pH reduces the formation of calcium phosphate, which leads to increased calcium absorption. Short-chain fatty acids also directly affect calcium absorption and increase its transport by modulating the signaling pathway.[52] Gastric SCFA infusions were used in a study in piglets, and the high concentration of SCFAs resulted in improved intestinal morphology, which, in turn, led to increased activity of digestive system enzymes and improved absorption of nutrients, vitamins, and minerals from the intestinal lumen into the blood. Unfortunately, intragastric SCFA infusions do not effectively improve the intestinal morphology in humans.[58] Another way to increase the count of SCFA-producing bacteria (including in humans) is to eat fats that contain palmitic acid esterified in the sn-2 position. In a study of rats, the intake of fat containing palmitic acid esterified in the internal position of triacylglycerols led to changes in the intestinal microbiome; for example, the number of bacteria that produce SCFAs increased, while the entire intestinal microflora profile remained unchanged.[59] There were similar results from a study of infants, in which esterified fatty acid in the sn-2 position was used in milk mixtures. The calcium-

saponified fat excretion was reduced, which indicated improved use of calcium and fat by the body.[60]

Equol. Once digested by enzymes, soy isoflavones can be absorbed by the intestinal villi, removed with the feces, or microbiologically fermented into equol. Equol is a metabolite of daidzein, a soybean aglycone. It exhibits strong estrogen-like effects and antioxidative activity, is very stable, and easily absorbed. Although all animal species studied so far can produce equol, only 20%–50% of humans have the microbiota suitable for its production. The difference in equol production between populations may result from eating habits. A much higher percentage of equol producers are vegetarian or Asian; people following these diets normally consume large amounts of soy.[61] Equol exhibits estrogen-like effects; thus, it protects bones from the loss of minerals by binding to estrogen receptors. Equol reduces bone resorption in postmenopausal women.[62] A study in mice showed that equol not only can prevent bone loss but also reduces inflammation, and so can be used to treat rheumatoid arthritis.[63] Because equol prevents the excessive resorption of minerals from bones, it affects the optimal secretion of PTH. Normal PTH levels in the body stimulate the synthesis of vitamin D_3 in the kidneys, which results in increased calcium absorption in the intestines. This mechanism shows how important estrogen-like compounds are for bone turnover and calcium metabolism in postmenopausal women.[21,22]

Serotonin. Serotonin is a neurotransmitter that regulates almost all brain functions. Recent studies have shown that serotonin may reduce bone formation. Specific strains of intestinal bacteria are involved in serotonin production. An in vitro study of osteoblast cells showed that serotonin activated 5-hydroxytryptamine 6 receptor (a G-protein–coupled serotonin receptor), which resulted in reduced alkaline phosphatase levels and inhibited bone mineralization. By contrast, the inhibition of this process in ovariectomized rats resulted in a significant increase in bone mass.[64]

Hypocalcemia is often observed in cows during lactation due to the increased demand for calcium. Serotonin regulates the blood calcium level; thus, intravenous injection of the serotonin precursor 5-hydroxy-1-tryptophan has been proposed to reduce the risk of hypocalcemia in animals. In a study, the calcium level decreased in control and intervention groups but increased in the cows receiving 5-hydroxy-1-tryptophan injections. On one hand, increased serotonin production by the intestinal microbiome may cause bone formation disorders; on the other, intravenous serotonin precursor reduces hypocalcemia.[65]

Lactobacilli. Lactobacillus reuteri is a probiotic strain of bacteria that may help increase intestinal calcium absorption. The bacteria can increase the serum calcidiol level and thus help increase intestinal calcium absorption by affecting the secretion of PTH. The bacteria are also likely to affect the activity of 25-hydroxylase, an enzyme necessary for the synthesis of vitamin D_3 in the liver.[52] Researchers also observed beneficial effects of *L. casei* and *L. acidophilus* on bone health in rats, not only increasing serum calcium level but also BMD in animals fed these bacteria.[66]

EXOGENOUS FACTORS

Dietary modifications

The bioavailability of calcium is affected by various factors provided with food. Macronutrients and micronutrients may improve or disorder calcium absorption. Table 2 lists major macronutrients and micronutrients.[67–82] Calcium absorption is affected by individual food ingredients and also by the specific diets consumed by people with specific needs, such as patients with hypertension, and athletes.

Fat. Fat has an impact on the bioavailability of fat-soluble vitamin D, which is necessary for calcium absorption.[67] Fat also contributes to the direct absorption of calcium, and the effect depends on the type of fatty acids it forms. The effectiveness of calcium absorption with 2 high-fat diets—a diet enriched in saturated or monounsaturated fatty acids and a low-fat diet—was assayed in an in vitro study; the higher pH with the low-fat diet was associated with a 90% lower bioavailability of calcium. The most effective absorption occurred in the presence of a diet with the addition of saturated fatty acids.[68] Although saturated fatty acids promote calcium absorption, their effect on bone health is less than that of a comparable proportion of monounsaturated fatty acids or a diet with a normal fat content. In that in vitro study, researchers found that the calbindine D9k intestinal gene expression and hepatic cytochrome P450 protein levels, and trabecular volume thickness were significantly higher in mice given food supplemented with monounsaturated fatty acids than in those given an unmodified diet. The recommended diet, therefore, is a one rich in monounsaturated fatty acids, because it has the most beneficial effect on bone health and a moderate increase in intestinal calcium absorption.[69]

Carbohydrates. Lactose. Dairy products are the dietary main source of both lactose and calcium.[83] Although

Table 2 **Nutritional factors affecting calcium absorption**

Nutrient	Item	Effect	Mechanism	References
Fat	Any type of fat	Increased calcium absorption	High vitamin D content in in high-fat foods	Maurya et al (2017)[67]
	SFA	Effective calcium absorption but low BMD	Low pH in the jejunum (reduced phosphate formation)	Bandali et al (2018)[68] Wang et al (2016)[69]
	Low-fat diet	90% lower calcium absorption	High pH in the small intestine (higher formation of phosphates)	Bandali et al (2018)[68]
	MUFA	Effective calcium absorption and high BMD	Low pH and much higher calbidin-d9k intestinal gene and trabecular volumes and thickness	Wang et al (2016)[69]
Carbohydrates	Lactose	Increased calcium absorption only in infants		Hodges et al (2019)[70]
	Fiber	Increased calcium absorption	Low pH in cecum	Albarracín et al (2016)[71]
		Reduction of β-glucuronidase activity	Reduction of estrogen reabsorption in the colon → reduction of BMD	Dai et al (2018)[72]
	Inulin and FOS	Increased calcium absorption	Changes in the balance of intestinal microflora and functional epithelial modifications	Krupa-Kozak et al (2016)[73]
Protein	CBP-Ca complex	Increased calcium absorption	Increased intestinal calcium permeability	Zhang et al (2018)[74]
	Desalted duck-egg-white peptides	Increased calcium absorption	Formation of calcium chelates in the lumen of the intestine and influence on regulation of enterocyte reproduction and their differentiation; → calcium chelates are well absorbed	Hou et al. (2017)[75]
		Phytic acid degradation	Increased calcium uptake in the presence of oxalate ions	
	Casein	Increased calcium absorption	Prevented the precipitation of calcium in the intestine	Sun et al (2018)[76]
Mineral ingredients	Calcium to phosphorus ratio (2:1)	Increased calcium absorption	Balance of proteins belonging to the system regulating bone remodeling (ie, OPG and RANKL)	Zhao et al (2019)[77]
	Iron	Impaired calcium absorption	Increased FGF-23 level in serum (for intravenous administration)	Fukao et al. (2018)[78]
			Impaired protein regulation: CB9k, PMCA1b, TRPV5, and TRPV6 in the intestine and decreased sodium-potassium ATPase activity (observed in patients with thalassemia)	Lertsuwan et al (2018)[79]
	Magnesium	Increased calcium absorption	Enzymes involved in vitamin D metabolism are magnesium dependent	Uwitonze et al. (2018)[80]
Oxalic acid		Impaired calcium absorption	Formation of insoluble calcium salts in the small intestine	Amalraj et al (2017)[81]
Phytic acids		Impaired calcium absorption	Formation of insoluble calcium chelates in the small intestine	
Caffeine		Reduced the activity of acid and alkaline phosphatase in serum	Lowering the rate of bone turnover	Xu et al (2019)[82]
		Increased blood calcium levels in the menopausal period	Decrease in calcium excretion in the kidneys	

Abbreviations: BMD, bone mineral density; CBP-Ca complex, calcium-binding peptide–calcium complex; FGF, Fibroblast growth factor; FOS, fructooligosaccharide; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

lactose alone does not affect calcium absorption, a diet low in dairy products is a predictor of calcium deficiency and, consequently, adversely affects bone metabolism.[70]

Fiber. Fiber reduces feces pH and lowers β-glucosidase activity in the large intestine. By acidifying the intestinal environment, calcium absorption may be increased by limiting phosphate formation.[71] The effect of dietary fiber intake on bone structure and metabolism seems to be sex dependent, the probable reason for which is that a decrease in β-glucuronidase activity in the large intestine leads to a decrease in estrogen reabsorption, which reduces BMD.[72]

Inulin. Inulin is a starch-like polysaccharide that acts as a prebiotic and allows changes in the balance of intestinal microflora and functional epithelial modifications. It increases the bioavailability of calcium in the intestine, mainly in combination with short-chain fructooligosaccharides.[73]

Protein. Calcium-binding peptides. An experimental study with rats determined that calcium-binding peptides from Pacific cod bones combined with calcium to increase the absorption and permeability of calcium in the intestine, improving bone's biomechanical properties, structure, and mineral density, while reducing the concentration of bone turnover markers in the blood.[74]

Desalted duck egg. The effect of desalted duck-egg-white peptides on calcium absorption was studied in the Caco-2 cell model. Using fluorescence spectrophotometry, it was determined that calcium chelates were formed as a result of the combination of the desalted duck-egg-white peptides with calcium ions. The interaction of desalted duck-egg-peptides with the transitional potential of the 6-channel calcium vanilloid receptor affects the regulation of enterocyte reproduction and their differentiation. The resulting calcium chelates are thus absorbed well in the intestines. Desalted duck-egg-white peptides also indirectly affect the efficiency of calcium and other mineral absorption; specifically, they counteract the effects of phytic acid by degrading it while also increasing calcium uptake in the presence of oxalate ions.[75]

Casein. During the digestion of casein (milk protein), casein phosphopeptides are released; in addition to their antimicrobial, anticariogenic, cytomodulatory, and immunomodulatory properties, casein phosphopeptides can transport calcium ions and increase their bioavailability.[84] In in vitro and in vivo studies, researchers have confirmed that casein phosphopeptides prevent the precipitation of calcium in the intestine, so its absorption is highly effective.[76] The monomer peptide P5, isolated from the mixture of casein phosphopeptides, has the greatest calcium-binding capacity; it can simultaneously bind 6 calcium ions.[85] Core-shell microparticles loaded with casein phosphopeptides in combination with chitosan oligosaccharides and tripolyphosphate are a promising ingredient in calcium supplements, because they gradually release ions, resulting in their prolonged uptake. Controlled release of calcium enhances its bioavailability in the intestines.[86]

Mineral ingredients. Phosphorus. The ratio of calcium to phosphorus in the diet should be in the range of 1.3:1 to 2:1. A significant increase or decrease in this ratio can lead to serious illnesses, such as impairment of growth, appetite, skin quality, or muscle strength; an inability to maintain correct posture; and, ultimately, even death. A diet low in phosphorus, even if supplied with appropriate amounts of calcium, leads to a decrease in apparent digestibility and renal excretion of phosphorus, whereas calcium excretion increases significantly; this may indicate the body is unable to use this mineral.[87] A 2:1 ratio of calcium to phosphorus is optimal for adequate bone growth and for maintaining the balance of osteoprotegerin and receptor activator of nuclear factor κ B ligand, which plays an important role in bone metabolism.[77]

Iron. Intravenous iron administration in people with a substantial deficiency of this mineral promotes an increase in the level of FGF-23, which leads to a decrease in intestinal calcium absorption. No such effect was observed in patients who used oral iron supplement.[78] Bone disease may develop in people with thalassemia (a disorder of hemoglobin synthesis due to a congenital defect in globin formation). This phenomenon is caused by impaired regulation of iron metabolism and its excessive absorption from the gastrointestinal tract. Intestinal absorption of calcium can be reduced by the impaired regulation of proteins associated with calcium transport (ie, calbindin-D9k, PMCA1b, TRPV5, and TRPV6). Another mechanism that may be responsible for impaired calcium absorption in the intestines in people with thalassemia is reduced activity of sodium–potassium ATPase in the intestines, which is responsible for the stabilization of intracellular sodium ion, whose presence is needed for the extrusion of absorbed calcium.[79]

Magnesium. Magnesium helps increase calcium absorption by altering the synthesis of vitamin D in the body.

The 3 main enzymes (24-hydroxylase and 1α-hydroxylase in the kidneys and 25-hydroxylase in the liver) that convert vitamin D into its active form are magnesium dependent. Magnesium deficiency causes calcidiol levels to decrease and PTH functioning deteriorates, which negatively affects calcium metabolism. Adequate magnesium supply is associated with a low risk of osteoporosis.[80]

Oxalic and phytic acids. Oxalic and phytic acids impair calcium absorption and thus also reduce BMD, leading to osteoporosis. However, vegetables with a low content of phytates and oxalates usually contain relatively small amounts of calcium.[81]

Caffeine. There is evidence that moderate caffeine consumption is good for health and reduces the risk of osteoporosis. Caffeine consumption has been observed to reduce the activity of acid and alkaline phosphatase in serum, which indicates slower bone metabolism and thus a reduction in the risk of osteoporosis. Although an increase in blood calcium levels (possibly by reducing calcium excretion) has been observed in an animal model of postmenopausal osteoporosis, moderate caffeine consumption in humans with adequate calcium intake does not significantly affect the metabolism of this mineral.[82] Moreover, osteoporosis is accompanied by oxidative stress, the occurrence of which may become a biomarker in the etiopathophysiology of the disease.[88] Coffee also is a good source of antioxidants. American (filtered) coffee has higher antioxidant capacity and higher phenolic content than Turkish coffee (boiled) and espresso (extracted under pressure).[89]

Diets. Dietary Approach to Stop Hypertension diet. The Dietary Approach to Stop Hypertension (DASH) diet is recommended because of its high content of fiber and low-fat dairy products and because following the diet can result in significantly reduced blood pressure. This diet is beneficial for the circulatory system and is recommended for people at risk of hypertension and its consequences, such as myocardial infarction. However, the effects of the DASH diet on bone health has not been fully investigated so far. Although the diet does not affect bone turnover biomarkers significantly, it substantially reduces blood calcitriol level. Users of the DASH diet have lower blood vitamin D_3 levels, which may deteriorate the skeletal structure and result in osteoporosis. Thus, this diet should be combined with an adequate supply of vitamin D_3 in food products or supplements.[90]

Low-calorie diet. A low-calorie diet is not recommended for people who play sports, because increased bone resorption may cause problems in the body's use of calcium. A 50% energy deficit caused an increase in the C-terminal telopeptide of type I collagen concentration and a decrease in the N-terminal propeptides of type I collagen level in women, indicating increased bone turnover.[91]

High-protein diet. Increasing the amount of protein in the diet is still controversial, and more research in humans and animals is needed to resolve the controversy.[92] In 1 short-term (10 days) study, consumption of a high-protein diet (2.1 g/kg body weight) by healthy women resulted in an increase in calcium absorption and reduction in calcium excretion, while reducing the rate of bone turnover.[8] However, when physically active women consumed a high-protein diet (2.2 g of protein per kilogram of body weight) for 6 months, no significant differences in bone density were observed.[93] In a study of postmenopausal women with osteoporosis, protein supplementation had beneficial effects on bone density, whereas increasing the dietary protein content had no effect.[94] Along with a high-protein diet, the body is also supplied with an increased amount of protein-related acids, the negative effect of which on calcium excretion is probably balanced by the beneficial effects on the skeleton that result from the increased consumption of protein.[95]

Plant-based diets. People who avoid meat and dairy products usually have low supplies of calcium and vitamin D and may be at high risk of osteoporosis. However, epidemiological studies have shown that vegetarians and vegans have often relatively high BMD levels. The main reason is the limited amount or absence of meat in their diet; meat, in addition to being high in protein, supplies the body with protein-related acids that promote lower bone density and increase the risk of fractures. Well-balanced vegetarian and vegan diets include appropriates amount of protein, calcium, and potassium, which positively affect bone health by reducing urinary calcium excretion.[96] A diet based on nuts, legumes, seeds, dark-green vegetables, added fats, eggs, low-fat milk, and fruit is beneficial to the developing skeletal system of young people.[97]

Pharmacologic treatment

Antiepileptic drugs. As mentioned previously, proton pump inhibitors reduce calcium absorption by limiting the secretion of hydrochloric acid in the stomach, which is necessary to dissolve calcium salts.[38] There are also

other groups of drugs that inhibit the bioavailability of calcium and thus increase the risk of osteoporosis. Antiepileptic drugs directly interfere with the absorption of calcium in the intestines and accelerate the catabolism of vitamin D_3 by inducing cytochrome P450 in the liver. Antiepileptic drugs also inhibit osteoblast growth, reduce the cellular response to PTH, and thus limit calcitonin secretion.[98]

Anticancer drugs. Anticancer therapy includes drugs that may cause calcium malabsorption. Menadione is the reference medicine for melanoma chemotherapy. Unfortunately, treatment is often unsuccessful because menadione is inactivated by intracellular glutathione. DL-buthionine-(*S,R*)-sulfoximine can be used to minimize melanoma-cell resistance because it reduces intracellular glutathione levels in cancer cells.[99] However, it also has the side effect of altering the expression of proteins involved in the transport of calcium ions from the intestinal lumen to the blood. As a result, calcium bioavailability is reduced.[100]

Diuretic drugs. Diuretics are often used to treat blood pressure because they facilitate sodium transport, which helps maintain fluid balance in the body. Unfortunately, these drugs also indirectly affect the transport of calcium ions and thus disrupt calcium reabsorption. The mechanism of action is closely related to the type of diuretic and may affect both paracellular and transcellular transport. For example, mannitol (an osmotic diuretic) increases the excretion of sodium, water, and (unintentionally) calcium, which are normally reabsorbed in proximal tubules through osmosis.[101]

Antituberculosis drugs. Vitamin D_3 deficiency is common in patients with tuberculosis. Isoniazid and rifampicin are drugs used to treat these patients, but they also affect vitamin D_3 metabolism, which is closely related to calcium metabolism. Antituberculosis drugs affect the activity of cytochrome P450, which is involved in the hydroxylation of vitamin D_3. According to recent reports, when rifampicin is used alone or in combination with isoniazid, the concentrations of 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 increase without affecting the active form of vitamin D_3 (1,25-dihydroxyvitamin D_3). At the same time, there is increased expression of the *CYP27A1* or *CYP2R1* genes (or both) belonging to cytochrome P450 family of genes, which are responsible for encoding hepatic 25-hydroxylase enzymes. The rifampicin and isoniazid combination inhibits CYP27B1 expression and stimulates CYP24A1 expression.[102]

Tetracycline antibiotics. Tetracycline antibiotics have a broad spectrum of activity and are used against gram-negative, gram-positive, and spirochete bacteria. They are prescribed for complications in the genitourinary system, respiratory system, gastrointestinal tract, and for serious and rare infections.[103] Tetracyclines reduce the absorption of calcium ions and other divalent and trivalent metals, and form insoluble chelates with them. Therefore, the consumption of food containing these metal compounds should be avoided during tetracycline therapy.[104]

Levothyroxine. Levothyroxine is a synthetic analogue of thyroxine, a thyroid hormone that regulates hypofunction of this endocrine organ. Levothyroxine-containing drugs should be taken on an empty stomach because levothyroxine interacts with dietary ingredients and other drugs.[105] Levothyroxine binds calcium carbonate in environments with a pH of 2, which reduces absorption of the drug and calcium ions by 20%–25%.[106] On the other hand, liquid levothyroxine limits drug sequestration by calcium and iron ions, which results in better bioavailability of both levothyroxine and metal ions.[107]

CONCLUSION

Many endogenous and exogenous factors affect the bioavailability of calcium. Some of these directly or indirectly increase calcium absorption, whereas others have a limiting effect. Calcium absorption can be improved by altering eating habits or by stimulating the quantitative or qualitative composition of the intestinal microflora with prebiotics and probiotics. Some medications, food ingredients, and dietary modifications decrease the calcium level and may lead to osteoporosis; to increase bone density and prevent osteoporosis, it is thus important to carefully design the diet with appropriate supplements that increase the bioavailability of calcium.

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Effects of Calcium Lactate-Enriched Pumpkin on Calcium Status in Ovariectomized Rats

Natalia Wawrzyniak [1], Anna Gramza-Michałowska [2], Ewa Pruszyńska-Oszmałek [3], Maciej Sassek [3] and Joanna Sulibursk [1,*]

[1] Department of Human Nutrition and Dietetics, Faculty of Food and Nutrition Science, University of Life Sciences, 60-624 Poznan, Poland; natalia.wawrzyniak@up.poznan.pl
[2] Department of Gastronomy Sciences and Functional Foods, Faculty of Food and Nutrition Science, University of Life Sciences, 60-624 Poznan, Poland; anna.gramza@up.poznan.pl
[3] Department of Animal Physiology and Biochemistry, Faculty of Veterinary Medicine and Animal Science, University of Life Sciences, 60-637 Poznan, Poland; ewa.pruszynska@up.poznan.pl (E.P.-O.); maciej.sassek@up.poznan.pl (M.S.)
* Correspondence: joanna.suliburska@up.poznan.pl

Abstract: This study aimed to evaluate the effects of enriched pumpkin on calcium status in ovariectomized rats. The study was conducted in sixty female Wistar rats, which were divided into six groups: a group fed a standard diet (C) and five ovariectomized groups fed a standard diet (OVX_C) or a diet with calcium lactate (CaL), with calcium lactate-enriched pumpkin (P_CaL), with calcium lactate and alendronate (CaL_B), or with calcium lactate-enriched pumpkin with alendronate (P_CaL_B). After 12 weeks of the intervention, the rats were sacrificed, and their blood and tissues were collected. The calcium concentrations in serum and in tissues were measured using flame atomic absorption spectrometry (AAS). Serum concentrations of procollagen type-1 amino-terminal propeptide (PINP), parathyroid hormone PTH, estrogen (ES), and osteocalcin (OC) were determined with enzyme-linked immunosorbent assay (ELISA). It was found that enriched pumpkin increased the calcium level in the kidneys (194.13 ± 41.01 mg) compared to the C (87.88 ± 12.42 mg) and OVX_C (79.29 ± 7.66 mg) groups. The addition of alendronate increased the calcium level in the femurs (267.63 ± 23.63 mg) and more than six times in the kidneys (541.33 ± 62.91 mg) compared to the OVX_C group (234.53 ± 21.67 mg and 87.88 ± 12.42 mg, respectively). We found that the CaL, P_CaL, and CaL_B groups had significantly lower PINP serum concentrations (4.45 ± 0.82 ng/mL, 4.14 ± 0.69 ng/mL, and 3.77 ± 0.33 ng/mL) and higher PTH serum levels (3.39 ± 0.54 ng/dL, 3.38 ± 0.57 ng/dL, and 3.47 ± 0.28 ng/dL) than the OVX_C group (4.69 ± 0.82 ng/mL and 2.59 ± 0.45 ng/dL, respectively). In conclusion, pumpkin enriched with calcium lactate affects calcium status and normalizes PINP and PTH serum levels in ovariectomized rats. Diet with enriched pumpkin and alendronate increase calcium concentration in the femur. Enriched pumpkin causes calcium to accumulate in the kidneys of ovariectomized rats; alendronate significantly exacerbates this effect.

Keywords: calcium; enriched pumpkin; postmenopausal osteoporosis; kidney accumulation

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1. Introduction

Epidemiological studies have shown an inadequate dietary calcium intake, which may increase the risk of developing osteoporosis, especially in postmenopausal women. Natural menopause and ovariectomy lead to decreases in estrogen level in the body, as well as malabsorption of calcium, and this contributes to bone loss [1–3]. The adequate supply and bioavailability of calcium are important for bone health, as are endogenous factors regulating the calcium metabolism. Among the hormones related to calcium metabolism and bone turnover are osteocalcin (OC), procollagen type-1 amino-terminal propeptide (PINP), and parathyroid hormone (PTH) [4–6]. PINP and OC are involved in bone and

dentin mineralization, which means that they are byproducts of the bone formation process. The release of osteocalcin from osteoblasts is stimulated by PTH, which is affected by serum calcium concentration, while PINP has become a reference marker in the diagnosis of osteoporosis, reflecting the process of collagen formation [5,6]. PTH is a hormone involved in calcium metabolism and bone remodeling. In the presence of sufficient serum calcium, extracellular calcium binds to receptors on parathyroid cells, reducing PTH levels. These mechanisms work in a feedback loop, meaning that PTH levels increase in hypocalcemia. The function of PTH is to increase serum calcium concentration though reabsorption from the kidneys, bone resorption, and intestinal absorption (by stimulating the production of active vitamin D in the kidneys) [7].

Bone loss characteristic of menopausal women is treated pharmacologically, mainly with bisphosphonates (e.g., alendronate) [8]. Alongside pharmacological therapy, a diet containing adequate amounts of good bioavailable calcium is part of osteoporosis treatment and prevention [9]. Dietary recommendations range from 1000 to 1500 mg of calcium/day, depending on age [10]. The most bioavailable calcium comes from dairy products; however, some people may suffer from lactose intolerance and cannot eat such kinds of products. Some plant products are also a good source of calcium, but it is less bioavailable [11]. Decreased intestinal absorption leads to the insufficient transport of calcium ions into the blood; thus, bioavailability is a critical factor that determines the effectiveness of calcium for bone development and health [12]. On the other hand, an excessive supply of calcium (mainly caused by taking calcium supplements) can result in many diseases, including renal failure, adrenal insufficiency, or hyperparathyroidism [13]. In order for the calcium contained in nutrients to be effectively absorbed in the intestines, factors that reduce its bioavailability should be avoided; these include iron, oxalic acid, and phytic acid. Conversely, factors that improve its bioavailability should be increased; these include vitamin D3, fructooligosaccharides, and inulin [14].

It is known that the bioavailability of calcium depends on its chemical form. Calcium lactate is an organic salt of calcium with a relatively high bioavailability and good solubility [15]. Calcium lactate is a component often used in supplements recommended for menopausal women [16,17]. An effective and easy way to increase the amount of calcium in the diet is through functional foods that contain much more calcium than natural foods [18]. One such innovative food is pumpkin enriched with calcium through osmotic dehydration using inulin, an osmotically active substance that increases calcium absorption [19]. Pumpkin is easy to use in an osmotic dehydration process leading to the enrichment of its tissues with calcium salts [19]. Moreover, pumpkin contains compounds that increase bone mineral density. Lutein is a carotenoid found abundantly in pumpkin which has been found to increase the mineral mass of bones, while suppressing their resorption by inhibiting the formation of osteoclasts [20,21]. Lutein also reduces the oxidative stress that accompanies postmenopausal osteoporosis [22]. Another carotenoid found in pumpkin is β-cryptoxanthin, which inhibits bone resorption and has an osteogenic effect through its effects on the expression of genes of proteins that are involved in bone formation [23]. Pumpkin might be widely used due to its low caloric content (average 26 kcal/100 g). Hypoglycemic and cardioprotective properties of pumpkin have been found; therefore, its consumption is recommended for people with hypertension, obesity, and diabetes. Moreover, due to the possibility of preparing dishes with a soft consistency, pumpkin is suitable both for infants, the elderly, and patients with gastrointestinal diseases [24].

Pumpkin has been enriched with calcium compounds using inulin, an osmotically active substance that increases calcium absorption, by changing the composition of the intestinal microbiota (increasing *Bifidobacterium*) [25], increasing the area of absorption in the cecum [26,27], and altering pH through the increased production of short-chain fatty acids [28]. It seems that consuming calcium-enriched pumpkin with inulin may be beneficial for people at high risk of osteoporosis development. Therefore, because pumpkin is a low-calorie food and a source of compounds with a high biological activity with a beneficial effect on the bone mineral status, and because it is easy to use in an osmotic

dehydration process, we believe that a combination of pumpkin with calcium lactate may be a good source of high bioavailable calcium in prevention and treatment of postmenopausal osteoporosis. This study, thus, aimed to determine the effects of calcium-enriched pumpkin on calcium status in an animal model of postmenopausal osteoporosis.

2. Materials and Methods

2.1. Materials and Reagents

Calcium lactate and inulin were purchased (Agnex, Białystok, Poland), as were pumpkins sourced from domestic organic farming. Experimental research and field studies on plants, including the collection of plant material, complied with relevant national, institutional, and international guidelines and legislation. Permission to collect the plant material (pumpkin) was obtained from the landowner. The ingredients of the animal feed—vitamins, minerals, L-cysteine, and choline—were purchased from Sigma-Aldrich (Darmstadt, Germany), while casein, corn starch, dextrin, cellulose, sucrose, and rapeseed oil were purchased from Hortimex (Konin, Poland). ELISA (enzyme-linked immunosorbent assay) kits were purchased from Mediagnost (Reutlingen, Germany).

2.2. Osmotic Dehydration

The pumpkin tissue was enriched with calcium lactate using a process of osmotic dehydration with inulin, an osmotically active substance. During osmotic dehydration, the exchange of components between the solution and the pumpkin takes place; the water is removed from the pumpkin and the compounds dissolved in the solution (inulin and calcium lactate) penetrate the pumpkin tissue. Thus, the purpose of osmotic dehydration of pumpkin was to saturate its tissues with calcium and inulin so that it became a source of these ingredients.

At first, the pumpkin was washed and cleaned, and the inner part attached to the pips was removed. The skin was then removed, and the pumpkin flesh was cut into cuboids (1 cm), which then underwent osmotic dehydration. Before the next stage, the pumpkin was frozen to −18 °C and stored for 24 h for further analysis. A 50% solution of inulin was prepared in small jars, containing 125 mL of distilled water and 125 g of inulin; calcium lactate was added to give a concentration of 5%. The frozen pumpkin cubes were added to this hypertonic solution at a ratio of 1:5 (50 g of pumpkin and 250 g of solution); the jars were tightly closed and shaken in a water bath heated to 50 °C for 2 h. After the osmotic dehydration, the supernatant solution was removed, and the pumpkin was filtered. The entire procedure was performed in three replications. Before the freeze-drying process, the pumpkin was frozen to between −18 °C and −28 °C for 24 h. The drained pumpkin was dried in a freeze dryer until the water content reached 3.5–5%. There was 2797.00 ± 11.90 mg of calcium in 100 g of the resulting lyophilizate. The calcium content in the lyophilizate was determined using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after mineralization and dilution with deionized water and LaCl3 (0.5%).

2.3. Animals

Twelve week old female Wistar rats were purchased from the Wielkopolska Center for Advanced Technologies, Adam Mickiewicz University (Poznań, Poland). The animals were housed under standard conditions: single-caged, with a 12 h light–dark cycle, acclimated for 1 week. This animal experiment was carried out in accordance with the guidelines for the care and use of laboratory animals.

2.4. Experimental Protocols

The study was conducted on sixty rats. All rats were fed the AIN-93 M diet [29]. The animals were randomized into six groups, with 10 animals in each group. At the start of the experiment, the total body weight of the rats did not differ between groups. Each rat was housed in a separate cage that was set up so that the rats could see each other, reducing the

stress associated with the study. At the start of the experiment, 50 rats were ovariectomized (OVX) to establish a rat model of postmenopausal osteoporosis.

After 7 days of convalescence, the nutritional intervention was started for 12 weeks. The control group (C) and one of the ovariectomized groups (OVX_C) received standard diet (no modification), the CaL group was fed standard diet with calcium carbonate replaced by calcium lactate, the P_CaL group was fed pumpkin enriched with calcium lactate, the CaL_B group was fed alendronate (a drug from the group of bisphosphonates) and calcium lactate, and the P_CaL_B group was fed alendronate and pumpkin enriched with calcium lactate. The standard diet included (per kg of diet) 465.7 g of cornstarch, 155 g of dextrin, 140 g of casein, 100 g of sucrose, 50 g of fiber, 40 g of sunflower oil, 35 g of mineral mix, 10 g of vitamin mix, 2.5 g of choline bitartrate, and 1.8 g of L-cysteine. The amounts of calcium lactate (27.22 g) and enriched pumpkin (180 g) in 1 kg of the diet were such that the calcium content was the same as in the standard diet. In the diets with bisphosphonate, the potassium alendronate amount was adjusted weekly as needed to maintain a dose of 3 mg per kilogram of body weight. The scheme of the experiment is shown in Figure 1.

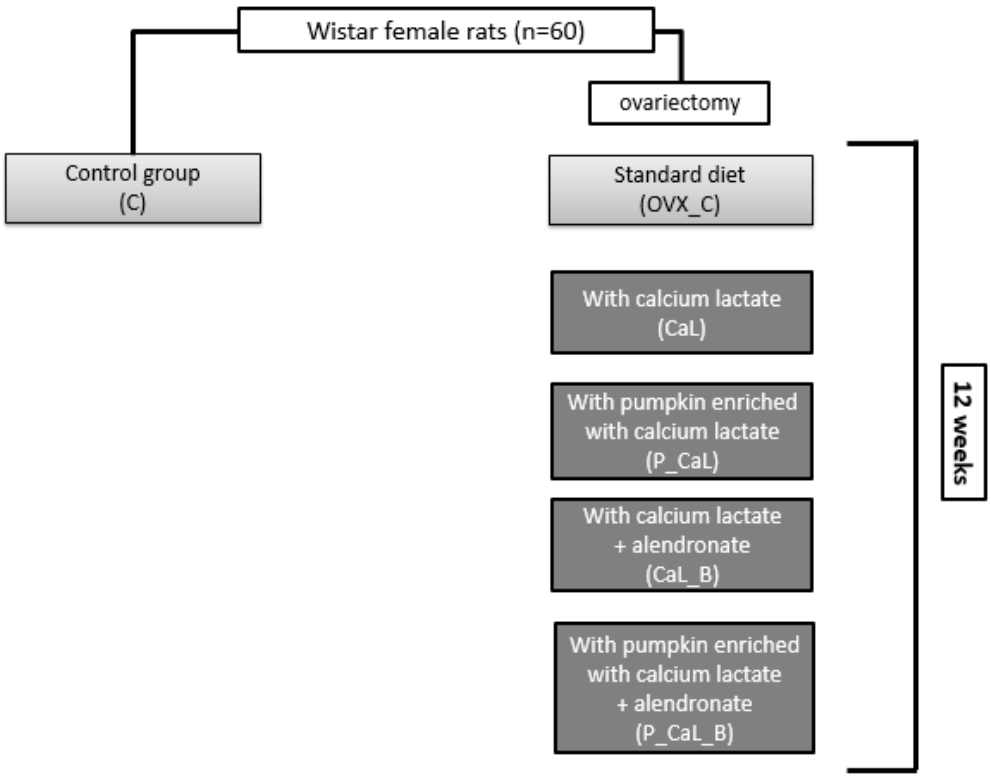

Figure 1. The scheme of the study. Groups shown in light-gray boxes were given the standard diet; the basic diet of the groups shown in the dark-gray boxes was a diet without calcium.

The animals were allowed to eat and drink deionized water ad libitum throughout the experiment. The rats in each group were weighed weekly, and food consumption was recorded daily. After completion of the experiment, body composition analysis of all animals was performed on a Bruker LF90II Body Composition Analyzer. The rats in each group were then decapitated, and blood and tissue samples were collected. Blood was centrifuged at 1200× *g* for 10 min at 4 °C. Livers, spleens, kidneys, pancreases, femurs, thigh muscles, and hair were removed, washed in saline, weighted, and kept at −80 °C for analysis. Hair was collected from the same anatomic area of each rat (the interscapular region).

2.5. Diet Analysis

The chemical composition of the diets included proteins, lipids, ash, carbohydrates, and fiber. The protein content was determined using the Kjeldahl method (AOAC, 1995; Foss Tecator, Höganäs, Sweden). The lipid content was determined using the Soxhlet method (PN-EN ISO 3947:2001; Soxtec System, Foss Tecator, Höganäs, Sweden). Ash content was determined after completely burning the sample in an oven (AOAC, 2000, Termo Fisher Scientific, Waltham MA, USA). Carbohydrate content was determined as 100% minus the percentage contributions of protein, fat, water, and ash. The fiber fractions were measured as total (TDF), insoluble (IDF), and soluble (SDF) dietary fiber content, following the enzymatic–gravimetric Asp method [30]. Other fiber fractions were evaluated using the Van Soest Assay [31] and are presented as neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (ADL), cellulose (ADC), and hemicellulose fractions (Fibertec, Foss Tecator, Höganäs, Sweden).

2.6. Calcium Analysis in the Diets

One gram samples of the diets were ashed in a muffle furnace at 450 °C until complete mineralization was achieved, and then dissolved in 1 mol/L nitric acid (Merck, Kenilworth, NJ, USA). The mineral content of the samples was determined using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after appropriate dilution with deionized water and LaCl3 (0.5%). The methods were validated by a simultaneous analysis of the reference material (Brown Bread BCR191, Sigma-Aldrich, St. Louis, MO, USA), with an accuracy of 92%.

2.7. Calcium Analysis in the Tissues

Each sample was mineralized in a Microwave Digestion system (Speedwave Xpert, Berghof, Eningen, Germany) by digesting in 65% (w/w) spectra pure HNO_3 (Merck, Kenilworth, NJ, USA). Deionized water was added and mixed after the digestion process. The mineral concentrations of the solutions were measured using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) after appropriate dilution with deionized water and $LaCl_3$ (0.5%) at a wavelength of $\lambda = 422.7$ nm. Verification used certified reference materials (Bovine liver 1577C, Sigma-Aldrich, Saint Louis, MO, USA) to determine the method's accuracy, which was found to be 91% for Ca.

2.8. Serum Parameters

Serum concentrations of PINP, PTH, ES, and OC were determined with ELISA and commercial kits (SunRed, Shanghai, China). Spectrometry was performed using an Infinite F50 spectrometer (Tecan Group Ltd., Männedorf, Switzerland).

2.9. Statistical Analysis

All results are presented as the means ± standard deviations. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK, USA). The Shapiro–Wilk test was used to determine the normality of the variable distributions. Statistical differences between the groups were assessed using one-way ANOVA followed by Tukey's post hoc test for normal distribution of values. However, Student's t-test was used for comparing two groups. A p-value < 0.05 was considered statistically significant.

3. Results

The diet compositions are presented in Table 1. The CaL_B and P_CaL_B diets were equivalent to the CaL and P_CaL diets and had the same nutritional composition. The higher content of carbohydrates in the diet of the control group and OVX_C was probably due to the higher content of cornstarch (cornstarch was replaced with calcium lactate and pumpkin in modified diets).

Table 1. The composition of the diets (mean and standard deviation).

Diet	Group		
	C/OVX_C	**CaL/CaL_B**	**P_CaL/P_CaL_B**
Caloric value (kcal/100 g)	326.37 ± 4.48	321.87 ± 3.37	322.27 ± 1.66
Carbohydrates (g/100 g)	47.92 ± 0.60 [b]	44.53 ± 0.90 [a]	45.44 ± 0.65 [a]
Fiber (g/100 g)	23.04 ± 0.60	25.05 ± 1.60	23.02 ± 0.41
Fat (g/100 g)	3.76 ± 0.41	4.33 ± 0.16	4.27 ± 0.02
Protein (g/100 g)	13.70 ± 0.21	13.67 ± 0.81	14.01 ± 0.59
Calcium (mg/g)	5.63 ± 0.37	5.68 ± 0.24	5.77 ± 0.15

C, control group; OVX_C, ovariectomized group; CaL, ovariectomized group with calcium lactate; CaL_B, ovariectomized group with calcium lactate and alendronate; P_CaL, ovariectomized group with pumpkin enriched with calcium lactate; P_CaL_B, ovariectomized group with pumpkin enriched with calcium lactate and alendronate; [a,b] significant differences between groups ($p < 0.05$).

We determined the effects of the modified diets on selected parameters. The results of the intervention are presented in Tables 2–5.

Table 2. Daily intake and parameters of the body composition analysis in rats (mean and standard deviation).

Parameter	Group					
	C	**OVX_C**	**CaL**	**P_CaL**	**CaL_B**	**P_CaL_B**
Daily intake of diet (g)	25.08 ± 0.63	25.11 ± 1.70	25.90 ± 0.55	24.31 ± 1.26	25.66 ± 2.29	24.84 ± 2.29
Daily intake of calcium (mg)	141.12 ± 3.56	141.30 ± 9.57	147.03 ± 3.11	140.31 ± 7.26	139.73 ± 12.44	145.01 ± 13.39
Body mass (g)	325.86 ± 25.97 [a]	421.90 ± 55.10 [b]	428.40 ± 51.1 [b]	384.11 ± 34.02 [ab]	433.30 ± 51.62 [b]	392.30 ± 34.88 [b]
Fat tissue (g)	122.08 ± 35.78 [a]	238.66 ± 55.07 [bc]	252.27 ± 47.47 [c]	167.79 ± 31.34 [ab]	267.13 ± 77.16 [c]	182.14 ± 37.70 [ab]

C, control group; OVX_C, ovariectomized group; CaL, ovariectomized group with calcium lactate; CaL_B, ovariectomized group with calcium lactate and alendronate; P_CaL, ovariectomized group with pumpkin enriched with calcium lactate; P_CaL_B, ovariectomized group with pumpkin enriched with calcium lactate and alendronate; [a,b,c] significant differences between groups ($p < 0.05$).

Table 3. The level of estradiol in serum of rats (mean and standard deviation).

Parameter	Group					
	C	**OVX_C**	**CaL**	**P_CaL**	**CaL_B**	**P_CaL_B**
ES ng/L	49.82 ± 4.62 [b]	21.68 ± 6.80 [a]	23.35 ± 2.58 [a]	20.05 ± 6.54 [a]	19.85 ± 2.50 [a]	18.12 ± 4.29 [a]

C, control group; OVX_C, ovariectomized group; CaL, ovariectomized group with calcium lactate; CaL_B, ovariectomized group with calcium lactate and alendronate; P_CaL, ovariectomized group with pumpkin enriched with calcium lactate; P_CaL_B, ovariectomized group with pumpkin enriched with calcium lactate and alendronate; ES—estradiol; [a,b] significant differences between groups ($p < 0.05$).

Table 2 shows the daily intake of diet and calcium and the results of body composition analysis. The daily intake was comparable in all groups. Ovariectomy significantly increased the bodyweight and fat content of the rats. It was observed that groups that received enriched pumpkin (P_CaL and P_CaL_B) showed significantly decreased fat content, compared to the other ovariectomized groups.

All the ovariectomized groups showed a significantly lower serum ES concentration compared to the control group, which was not altered by the addition of calcium lactate or enriched pumpkin (Table 3). The obtained estrogen levels in groups confirmed the decreased concentration of estrogen after ovariectomy.

Table 4. Calcium content in the serum and tissues (mean and standard deviation).

Tissue	Group					
	C	**OVX_C**	**CaL**	**P_CaL**	**CaL_B**	**P_CaL_B**
Serum (ug/mL)	133.04 ± 11.91 ^{ab}	121.80 ± 8.10 ^{ab}	115.58 ± 6.23 ^{a}	139.10 ± 9.84 ^{b}	122.89 ± 12.99 ^{ab}	127.51 ± 17.27 ^{ab}
Femur (mg/g dm)	234.53 ± 21.67 ^{ab}	217.48 ± 7.24 ^{a}	231.98 ± 60.74 ^{ab}	235.80 ± 13.37 ^{ab}	254.54 ± 19.15 ^{ab}	267.63 ± 23.63 ^{b}
Pancreas (ug/g dm)	108.81 ± 11.46	113.75 ± 10.03	128.72 ± 19.30	106.66 ± 26.70	116.85 ± 11.69	107.56 ± 21.92
Hair (ug/g dm)	574.21± 75.87 ^{d}	459.45 ± 50.19 $^{c,\#}$	304.27 ± 39.59 ^{a}	382.73 ± 49.75 $^{b,\#}$	389.72 ± 49.98 ^{b}	382.41 ± 23.11 ^{b}
Spleen (ug/g dm)	530.71 ± 109.98 ^{bc}	470.30 ± 99.57 $^{b,\#}$	592.83 ± 39.82 ^{c}	450.19 ± 89.14 $^{b,\#}$	291.36 ± 46.23 $^{a,\#}$	316.06 ± 44.92 ^{a,*}
Liver (ug/g dm)	158.08 ± 10.47 ^{cd}	139.58 ± 11.83 ^{bc}	132.82 ± 16.54 ^{ab}	117.59 ± 9.25 $^{a,\#}$	161.20 ± 19.83 $^{d,\#}$	169.85 ± 11.64 ^{d,*}
Heart (ug/g dm)	119.67 ± 14.73 ^{c}	81.90 ± 19.50 ^{b}	80.44 ± 12.5 ^{b}	75.93 ± 8.01 ^{b}	75.05 ± 7.31 ^{b}	53.80 ± 6.63 ^{a,*}
Brain (ug/g dm)	182.33 ± 31.12	221.45 ± 81.06	177.15 ± 78.82	219.57 ± 97.63	209.70 ± 77.67	181.34 ± 28.7
Muscle (ug/g dm)	49.13 ± 8.25 ^{cd}	52.23 ± 10.86 $^{d,\#}$	36.25 ± 10.35 ^{bc}	34.88 ± 9.30 ^{b}	21.58 ± 9.68 $^{a,\#}$	18.67 ± 5.82 ^{a,*}
Kidney (ug/g dm)	87.88 ± 12.42 ^{a}	79.29 ± 7.66 $^{a,\#}$	123.92 ± 23.58 ^{a}	194.13 ± 41.01 $^{b,\#}$	477.78 ± 80.4 $^{c,\#}$	541.33 ± 62.91 ^{d,*}

C, control group; OVX_C, ovariectomized group; CaL, ovariectomized group with calcium lactate; CaL_B, ovariectomized group with calcium lactate and alendronate; P_CaL, ovariectomized group with pumpkin enriched with calcium lactate; P_CaL_B, ovariectomized group with pumpkin enriched with calcium lactate and alendronate; dm, dry mass; ^{a,b,c,d} significant differences between groups ($p < 0.05$); # significantly different in comparison to Ca_L group; * significantly different in comparison to P_CaL group.

Table 5. Parameters of calcium and bone metabolism in serum of rats (mean and standard deviation).

Parameter	Group					
	C	**OVX_C**	**CaL**	**P_CaL**	**CaL_B**	**P_CaL_B**
PINP (ng/mL)	3.25 ± 1.10 ^{a}	4.69 ± 0.82 ^{c}	4.45 ± 0.82 ^{ab}	4.14 ± 0.69 ^{ab}	3.77 ± 0.33 ^{ab}	4.62 ± 0.54 ^{c,*}
PTH (ng/dL)	3.35 ± 0.5 ^{b}	2.59 ± 0.45 $^{a,\#}$	3.39 ± 0.54 ^{b}	3.38 ± 0.57 ^{b}	3.47 ± 0.28 ^{b}	2.80 ± 0.35 ^{ab}
OC (ng/mL)	18.91 ± 3.59 ^{b}	16.23 ± 1.08 ^{ab}	15.61 ± 3.38 ^{ab}	14.53 ± 4.40 ^{ab}	12.78 ± 4.70 ^{a}	11.70 ± 3.04 ^{a}

C, control group; OVX_C, ovariectomized group; CaL, ovariectomized group with calcium lactate; CaL_B, ovariectomized group with calcium lactate and alendronate; P_CaL, ovariectomized group with pumpkin enriched with calcium lactate; P_CaL_B, ovariectomized group with pumpkin enriched with calcium lactate and alendronate; PINP, procollagen type I N propeptide; PTH, parathyroid hormone; OC, osteocalcin; ^{a,b,c} significant differences between groups ($p < 0.05$); # significantly different in comparison to Ca_L group; * significantly different in comparison to P_CaL group.

Table 4 presents the calcium concentrations in the serum and tissues by experimental group. When all groups were compared, we observed that ovariectomy did not affect the serum calcium concentration, but that the P_CaL group had a significantly higher calcium level than did the CaL group. Ovariectomy slightly decreased the calcium content in femurs, while the group receiving enriched pumpkin and alendronate (P_CaL_B) showed a significant increase in calcium content. It was found that ovariectomy significantly decreased calcium concentration in the heart and hair in rats, and that this effect was intensified by the other modified diets. Ovariectomy did not significantly affect the calcium content of the spleen or the liver. Calcium lactate markedly increased the concentration of calcium in the spleen, and the addition of the drug significantly lowered the concentration of this parameter in comparison to other groups. The enriched pumpkin significantly reduced the calcium content of the liver, while rats with alendronate had the highest levels of calcium in the liver of any of the groups. While ovariectomy did not alter muscle calcium content, calcium lactate and enriched pumpkin reduced it significantly, and the addition of the drug intensified this effect. Ovariectomy did not affect the calcium content of the kidneys. Groups P_CaL, CaL_B, and P_CaL_B showed significantly higher calcium levels in the kidney than did other groups. Enriched pumpkin more than doubled the calcium level in kidneys in comparison to the C and OVX_C groups. Moreover, alendronate increased the calcium level in kidneys by a factor of more than six, compared with the

OVX_C group. In this study, we compared the OVX_C and CaL groups, which differed by the addition of calcium salt to the standard diet. Calcium lactate in the standard diet (CaL) significantly decreased calcium in the hair and muscle while increasing it in the spleen and kidneys, compared to the OVX_C group. Rats receiving the enriched pumpkin diet (P_CaL) had significantly higher calcium levels in the hair and kidney and markedly lower calcium levels in the spleen and liver than did the CaL group. The addition of alendronate to both the calcium lactate diet and to the enriched pumpkin diet increased the calcium level in femurs (although markedly only in the P_Ca_B group), livers, and kidneys, while decreasing calcium level in hearts and muscles, as compared to the CaL and P_CaL groups.

Table 5 shows the levels of the parameters involved in calcium and bone metabolism. We observed that ovariectomy increased the serum concentration of PINP. On the other hand, the modified diets led to a decrease in the serum PINP concentration, except for in the P_CaL_B group. Ovariectomy decreased serum PTH concentration, while all the modified diets increased it to values similar to those found in the control group. Ovariectomy, calcium lactate, and enriched pumpkin did not significantly affect the level of OC, while the addition of the drug reduced it, compared to the control group.

4. Discussion

It was generally observed that, in the femurs and kidneys of the rats fed fortified pumpkin, the levels of calcium were high, while most of the other organs (pancreas, spleen, heart, and muscles) contained low levels of the mineral. This may indicate a shift of calcium ions from other tissues to the bones, mostly to the kidneys, with consequent accumulation. We observed, in the P_CaL_B group, a significant decrease in thigh muscle calcium, while femur calcium was significantly higher than in the OVX_C group. This may indicate a shift of calcium from the thigh muscle to the bone attached to this muscle, balancing bone calcium content. Interactions between muscles and bones have been confirmed by previous studies showing that the communication between these is not only mechanical, but also acts through the secretion of biochemical factors and minerals [32–34]. The results of those studies confirmed that alendronate inhibits bone resorption in the postmenopausal condition and reduces the loss of calcium from bone, at the expense of decreasing calcium in other organs [35,36]. A probable mechanism of the increased calcium content in the tissues after the consumption of fortified pumpkin is enhanced absorption of calcium by inulin, which was a component of the enriched pumpkin. The large influx of ions into the body is then distributed to the tissues bearing the CaSR (calcium-sensing receptor) cells. Through this receptor, calcium ions from the external environment pass into the internal, thus increasing the calcium content in the tissues [12,37]. The change in tissue distribution of calcium ions was likely the reason for the observed low levels of calcium in the liver in the enriched pumpkin group compared to the calcium lactate group. The group enriched with pumpkin had a relatively low concentration in the liver and pancreas, but a high calcium content in the kidneys, which may indicate a shift of calcium ions and its increased excretion and increased reabsorption in the kidneys.

However, in the present study, we saw both beneficial and detrimental effects of pumpkin enrichment on the calcium status of ovariectomized rats. The greatly increasing calcium concentration in the kidneys with the consumption of enriched pumpkin was unexpected and could lead to serious complications in the body. Calcium accumulation in the kidneys may be associated with increased excretion of this mineral, although, in this study, the calcium concentration of the animal's urine was not considered; this may limit the clear interpretation of the phenomenon. Our results may indicate possible side-effects of the components of the calcium-enriched pumpkin, such as inulin which, despite increasing the absorption of calcium from the intestines [28], also contributes to its increased excretion [38]. Calcium accumulation in the kidneys can lead to kidney dysfunction, causing kidney stones [39] and calcification of soft tissues [10] and blood vessels [40]. The main components of kidney stones are calcium oxalate (approximately 80%) and calcium phosphate (about 15%). The consequences of untreated kidney stones may include

inflammation of the urinary system and chronic kidney disease [41]. Nephrolithiasis is also a risk factor for osteoporotic fractures. The kidneys play an important role in the homeostasis of calcium, which is filtered and reabsorbed in this organ. If calcium is improperly filtered or reabsorbed, it causes hypercalciuria and a decrease in serum calcium, leading to the increased release of calcium from bones to the plasma. BMD consequently decreases, and osteoporosis can develop [42]. Some other components of pumpkin which may affect renal function are vitamins A and E [24]. Rats with pumpkin in their diet had definitely higher supply of vitamins A and E than did those receiving the standard diet, and excessive amounts of these vitamins can lead to glomerular hyperfiltration and hypercalcemia [43]. The biologically active form of vitamin A is retinoic acid, which can lead to the progression of glomerular disease [44,45]. However, in this study, the vitamin levels in blood and tissues were not measured; hence, the above explanation for the negative effects of fortified pumpkin on the kidneys is only speculation. In studies by other authors, pumpkin has a rather positive effect on the kidneys [46–48]. The present study may indicate that enriched pumpkin increases the concentration of calcium in the kidneys. It is possible that the glomeruli had become damaged by the excessive saturation of the body with vitamins, impairing calcium reabsorption. Similar differences were noted with respect to calcium concentration in the kidneys in the CaL group. Inulin is also responsible for the increased retention of calcium in the bones, which affects the accumulation of calcium, magnesium, and iron in the bones, but only when the demand for minerals is high, such as during growth [26].

Another component of the rats' diet that affects calcium metabolism is alendronate, the main bisphosphonate drug. Alendronate is widely used in women with osteoporosis, where it is administered orally in weekly doses. There are many studies on the prevention of bone fractures in patients using alendronate [49]. Bisphosphonates, through coordinating between the calcium ions of the crystal structure and the phosphonate groups, bind specifically with hydroxyapatite [50]. Alendronate works by inhibiting bone resorption through the induction of osteoclast apoptosis. Alendronate also stimulates osteoblast differentiation and alleviates the apoptosis of osteoblasts and osteocytes [51]. However, bisphosphonates should not be used for long periods, as they have numerous side-effects, including kidney damage. The strong affinity of alendronate for calcium ions, soluble calcium, and insoluble calcium causes the formation of aggregates and complexes [52], which may be retained in the kidneys, damaging renal tubules and causing them to undergo necrosis [53]. Although nephrotoxicity is mainly an issue for intravenous bisphosphonates (oral medications usually do not show similar side-effects), there are some exceptions. In one study [54], significant increases in kidney calcium levels were observed in rats fed a diet supplemented with alendronate; this may indicate adverse calcium accumulation in the kidneys. We can only assume that there were changes in the kidneys of the rats (such as kidney stones and calcification) receiving enriched pumpkin or alendronate, as we unfortunately do not have results to support this speculation. In the future, we plan to extend the analysis to include the biochemical and histopathological parameters of kidney function. An association between natural menopause and surgical menopause with a higher risk of an incident kidney stone has been found in clinical studies [55]. It, thus, seems that calcium-enriched pumpkin, especially when combined with alendronate, could increase the risk of this kind of renal dysfunction in menopausal women.

We also observed an effect of the enriched pumpkin on the hormonal balance of calcium metabolism. Apart from obvious hormonal changes, such as the decrease in ES concentration after ovariectomy, an increase in PINP concentration after ovariectomy was also observed, which may indicate an increase in bone formation and, thus, bone turnover. On the other hand, in the groups with the modified diets, ovariectomy led to a smaller increase in PINP concentration. PINP is a marker of bone formation, and, in the absence of estrogen, its concentration increases, which leads to more pronounced bone turnover [56,57]. In our study, the modified diets produced an effect similar to that of administration of ES, except for in the P_CaL_B group. The opposite effect on PINP seen in the P_CAL_B

group may be due to the interaction between alendronate and enriched pumpkin. This phenomenon requires further research. We moreover found that ovariectomy reduced the concentration of PTH and OC, while modified diets normalized the PTH level in the ovariectomized rats. It is known that PTH concentration depends on the amount of calcium in the blood. However, our results did not confirm this. Modified diets may have impacted other biochemical or hormonal factors associated with the experimental parameters.

Pumpkin enriched with calcium lactate also affects body composition. In a previous study, it was found that calcium lactate-enriched pumpkin decreased serum leptin levels, thereby lowering body fat and weight in ovariectomized rats [58].

Limitations

This study had a number of limitations which may have affected the results. The study did not involve a sham group; therefore, the effect of sham surgery could not be taken into account. However, we did compare the effect of ovariectomy with an unoperated control group. We did not consider indicators of oxidative stress, antioxidant status, or vitamins. The composition of the diet was not analyzed in detail (e.g., vitamin D level was not measured). Moreover, the rats' urine was not collected, and parameters of renal function were not noted; these might have broadened the interpretation and affected our discussion of the results.

5. Conclusions

Pumpkin enriched with calcium lactate affects calcium status and normalizes PINP and PTH serum levels in ovariectomized rats. A diet enriched with pumpkin and alendronate increases calcium concentration in the femur. Calcium lactate-enriched pumpkin causes calcium to accumulate in the kidneys of ovariectomized rats. Alendronate significantly exacerbates this adverse renal effect. Our results, thus, point to possible side-effects of using calcium lactate-enriched pumpkin.

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ORIGINAL ARTICLE

Calcium carbonate-enriched pumpkin affects calcium status in ovariectomized rats

Natalia Wawrzyniak[1] · Anna Gramza-Michałowska[2] · Paweł Kurzawa[3,4] · Paweł Kołodziejski[5] · Joanna Suliburska[1]

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Abstract Calcium carbonate ($CaCO_3$)-enriched pumpkin may serve as a good source of calcium for patients diagnosed with osteoporosis. In this study, we aimed to determine the effect of $CaCO_3$-enriched pumpkin on Ca status in ovariectomized rats. The study included 40 female Wistar rats divided into five groups (n = 8). One group was fed with a standard diet (control group), while the other four groups were ovariectomized and received a standard diet (control ovariectomized group), or a diet containing $CaCO_3$-enriched pumpkin, alendronate, or both. The nutritional intervention lasted 12 weeks, and then the rats were euthanized. Tissue and blood samples were collected and assessed for the levels of total Ca, estradiol, parathyroid hormone, and procollagen type I N propeptide. In addition, a histological analysis was performed on femurs. The results of the study suggest that $CaCO_3$-enriched pumpkin can increase Ca content in femurs and improve bone recovery in ovariectomized rats. Furthermore, enriched pumpkin contributes to Ca accumulation in the kidneys, and this effect is more pronounced in combination with alendronate.

Keywords Calcium · Enriched pumpkin · Osteoporosis · Ovariectomy · Rat

Abbreviations

PINP	Procollagen type I N propeptide
OC	Osteocalcin
PTH	Parathyroid hormone
ES	Estradiol
Ca	Calcium
$CaCO_3$	Calcium carbonate
ELISA	Enzyme-linked immunosorbent assay

Introduction

Postmenopausal osteoporosis is a condition characterized by a reduction in bone mineral mass due to a decline in estrogen levels as a result of endocrine disruption of the ovaries (Black and Rosen 2016). It is mainly diagnosed using dual X-ray absorptiometry (Yong and Logan 2021), but serum bone markers are also considered one of the prognostic indicators to determine disease development or treatment effects (Kanis et al. 2020). Bone turnover markers can be divided into two groups: formation markers and resorption markers. The former includes PINP and OC, which are by-products of bone mineralization. PINP found in the serum is released during collagen formation, while PTH stimulates osteoblasts to release OC (Marcu et al. 2011; Greenblatt et al. 2017). Histopathological analysis of bone aids in understanding the bone structure and cellular changes occurring in the bone

✉ Joanna Suliburska
jsulibur@up.poznan.pl

1 Department of Human Nutrition and Dietetics, Faculty of Food Science and Nutrition, Poznań University of Life Sciences, 31 Wojska Polskiego St., 60-624 Poznan, Poland

2 Department of Gastronomy Science and Functional Foods, Faculty of Food Science and Nutrition, Poznań University of Life Sciences, Wojska Polskiego 31, 60-624 Poznan, Poland

3 Department of Clinical Pathology, Poznań University of Medical Sciences, Przybyszewskiego 49, 60-355 Poznan, Poland

4 Department of Oncological Pathology, Pozna University of Medical Sciences, Szamarzewskiego 84, 60-596 Poznan, Poland

5 Department of Animal Physiology, Biochemistry and Biostructure, Faculty of Veterinary Medicine and Animal Science, Poznań University of Life Sciences, Wojska Polskiego 28, 60-637 Poznan, Poland

tissue, thereby confirming the presence or absence of osteoporotic changes. A fewer number of osteoblasts and osteocytes (forming cells) and an increased number of osteoclasts (resorbing cells) might indicate the presence of osteoporosis. Moreover, a higher ratio of fat bone marrow to bone marrow cellularity is a negative prognostic indicator of osteoporosis development (Marcu et al. 2011). The percentage of de novo-built bones is indicated by the percentage of woven bones. First, a woven bone (immature bone undergoing reconstruction) is formed from mesenchymal osteoblasts; the woven bone is then remodeled into a lamellar bone (mature bone that does not undergo transformation) from surface osteoblasts, a process common in the general population. However, the proportion of woven bone to lamellar bone varies among individuals (Shapiro and Wu 2019). The number of woven bones is generally high during recovery from injury or during growth in children, whereas in adults bone formation and resorption processes occur continuously and bones undergo standard transformations (Downey and Siegel 2006).

The diagnosis of osteoporosis should be followed by appropriate treatment to increase bone density and decrease bone turnover. Pharmacological treatment intended for osteoporosis involves the use of drugs that reduce bone resorption and/or accelerate bone formation, such as bisphosphonates (alendronate and risedronate), denosumab, and teriparatide, or hormone replacement (Gallagher and Tella 2014). However, these drugs can cause side effects when used for a long term; for example, the use of bisphosphonates for over two years can result in atypical bone fractures (Black and Rosen 2016), jaw necrosis (Shibahara 2019), or digestive disorders (Fadda et al. 2015). Therefore, the public health system is currently focusing on developing new approaches for the treatment and prevention of osteoporosis.

A diet containing adequate amounts of Ca with high bioavailability is essential for maintaining bone health, as Ca constitutes a large portion of bone mass (Weaver 2015). In addition to eliminating substances that can reduce Ca absorption (e.g. phytic or oxalic acid), components that increase Ca bioavailability (e.g. vitamin D and inulin) should be included adequately in the diet (Wawrzyniak and Suliburska 2021), in order to improve bone health. Endogenous factors regulating the metabolism of Ca are equally important. The concentration of Ca in the blood is regulated mainly by active vitamin D (1,25-dihydroxycholecalciferol), calcitonin, and PTH. PTH plays a major role in Ca regulation in the blood and bone turnover, which stimulates the release of Ca in bones and its reabsorption in kidneys. In addition, PTH stimulates the synthesis of vitamin D, which increases intestinal Ca uptake, and inhibits collagen synthesis by osteoblasts. Collagen is the organic matrix for minerals (including Ca) deposited in bone. On the contrary, calcitonin inhibits bone resorption, the activation of vitamin D, and Ca renal reabsorption. Therefore, the status of Ca in the body is influenced by the supply, bioavailability, and factors regulating its metabolism (Marcu et al. 2011; Greenblatt et al. 2017).

Epidemiological studies have shown that Ca deficiency is common worldwide, and there is a need to identify dietary sources with high bioavailable Ca (Balk et al. 2017). It has been found that Ca-enriched food products based on pumpkin can help overcome this challenge (Weaver and Liebman 2002). Pumpkin comprises compounds with high biological activity, such as carotenoids, including α-carotene, β-carotene, zeaxanthin, or lutein, which have a beneficial effect on the bone mineral status, reduce susceptibility to fractures, and prevent the progression of osteoporosis (Kulczynski and Gramza-Michałowska 2019). Moreover, pumpkin is easy to use in an osmotic dehydration process, which allows the enrichment of its tissues with Ca salts (Kulczyński et al. 2020). Enriched pumpkin contains inulin which increases Ca bioavailability(Bakirhan and Karabudak 2021), other ingredients that can improve bone health, including lutein (Takeda et al. 2017; Tominari et al. 2017) and β-cryptoxanthin, and a pigment that inhibits bone resorption and reduces oxidative stress (Yamaguchi 2012; Ozaki et al. 2015). Pumpkin has also been used in previous studies due to its low caloric content (average 26 kcal/100 g). Moreover, pumpkin exhibits cardioprotective and hypoglycemic properties, and is therefore recommended for diabetics and patients with arterial hypertension and obesity (Kulczynski and Gramza-Michałowska 2019).

According to the available data, the ingredients of Ca-enriched pumpkin can increase bone mineral density and thus reduce the risk of osteoporosis. Therefore, this study aimed to determine the effect of Ca-enriched pumpkin on Ca metabolism and status in ovariectomized rats.

Methods

Materials and reagents

Pumpkins (*Cucurbita maxima*, yellow melon) were obtained from a domestic organic farm after seeking permission from the land owner. Experimental research and field studies, including the collection of plant materials, were conducted in accordance with relevant institutional, national, and international guidelines and legislation. Inulin and $CaCO_3$ were purchased from Agnex (Białystok, Poland). Sucrose, rapeseed oil, dextrin, corn starch, and casein were obtained from Hortimex (Konin, Poland). Minerals and vitamins were procured from Sigma-Aldrich (Darmstadt, Germany). ELISA kits were purchased from SunRed (Shanghai, China).

Osmotic dehydration

Pumpkins were enriched with $CaCO_3$ by osmotic dehydration with inulin, an osmotically active substance. Then, they were cleaned and washed, and the inner part attached to the seeds was removed. Subsequently, the skin was peeled, and the pumpkins were cut into cubes (1 cm) and frozen for 24 h at − 18 °C. After freezing, a solution composed of inulin (125 g) and distilled water (125 ml) in a 1:1 ratio was prepared in small jars. $CaCO_3$ was added to the prepared solution such that its content was 5% of the solution. Next, 50 g of frozen pumpkin cubes (5:1) was added to 250 g of the hypertonic solution in the jars. The jars containing the pumpkin cubes were closed and shaken for 2 h at 50 °C in a water bath. Upon completion of osmotic dehydration, the solution formed above the pumpkin cubes was removed and the cubes were filtered. This process was repeated three times. Then, the pumpkin cubes were frozen at − 18 to − 28 °C for the next 24 h, and freeze-dried to 3.5–5% water content. A 100 g of the obtained lyophilizate contained 2390.8 ± 63.3 mg of Ca (Kulczyński et al. 2020) compared to nonenriched freeze-dried pumpkin, in which the Ca content was only 264.89 ± 0.59 mg/100 g (Kulczynski and Gramza-Michałowska 2019).

Animals

Forty female Wistar rats aged 12 weeks were purchased from the University of Adam Mickiewicz in Poznań, Greater Poland Center for Advanced Technologies, Poland. The animals were allowed to acclimatize for 1 week and then housed individually in cages under a 12-h dark–light cycle. All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. Approval for the study was obtained from the Local Ethics Committee in Poznań (protocol number: 34/2019). The reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Experimental protocols

Throughout the experiment, the rats were fed with the standard AIN-93 M diet (Reeves and Suppl 1997). The animals were divided into five groups, with eight in each. At the beginning of the experiment, the body weight of the rats was measured and found to be similar. Four groups (32 rats) were ovariectomized. After a one-week recovery period, the rats were subjected to a 12-week nutritional intervention. Unmodified standard AIN-93 M diet was given to the control group (C) and to one of the ovariectomized groups (OVX_C), while the other three groups received a diet containing $CaCO_3$-enriched pumpkin (OVX_P group), alendronate (OVX_B), or alendronate and $CaCO_3$-enriched pumpkin (OVX_P_B group). The standard diet contained $CaCO_3$ as a source of Ca. Figure 1 presents a schematic of the experiment.

The amount of enriched pumpkin added to the modified diet was such that the Ca content of the modified diet was the same as that of the standard diet. For the OVX_B and OVX_P_B groups, the amount of alendronate was adjusted weekly, ensuring that they received 3 mg/kg body weight. All animals were allowed *ad libitum* access to deionized water and feed. The intake by animals was recorded daily, and the body weight was measured weekly. At the end of the experiment, a body weight was measured, and then the rats were decapitated. Blood samples were collected and stored at − 80 °C. Serum was obtained by centrifuging the blood samples at 1200 × g for 10 min at 4 °C. The femurs, liver, kidneys, femoral muscles, spleen, and pancreas were isolated for analyses. The obtained tissues were washed with saline, weighed, and stored at − 80 °C. Hair was collected from the interscapular area.

Diet analysis

The lipid content in the samples was determined using the Soxhlet method (PN-EN ISO 3947:2001; Soxtec System, Foss Tecator), while the protein content was determined using the Kjeldahl method (AOAC, 1995; Foss Tecator).

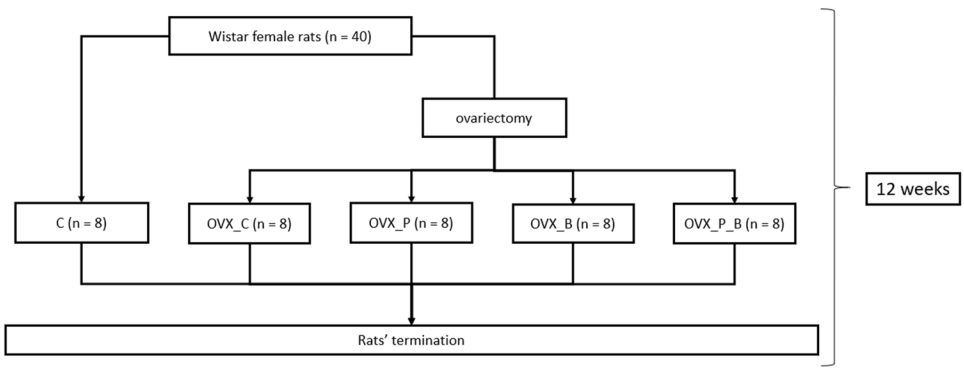

Fig. 1 Scheme of the study. C, control group receiving standard diet; OVX_C, ovariectomized group receiving standard diet; OVX_P, ovariectomized group receiving diet with pumpkin enriched with CaCO3; OVX_B, ovariectomized group receiving diet with alendronate; OVX_P_B, ovariectomized group receiving diet with pumpkin enriched with $CaCO_3$ and alendronate

The sample was completely burned in a muffle furnace to determine the ash content (AOAC, 2000). The carbohydrate content was calculated from the content of fat, protein, water, and ash. The total fiber fraction was measured using the enzymatic-gravimetric method (Dziedzic et al. 2012).

Ca analysis in diets

To determine Ca content in diets, 1 g of each sample of diet was burned in a muffle furnace at 450 °C until mineralization. The samples were then dissolved in 1 mol/l nitric acid (Merck, Kenilworth, NJ, USA). Using flame atomic absorption spectrometry, the mineral content was determined after diluting the samples with appropriate amounts of $LaCl_3$ (0.5%) and deionized water (AAS-3, Carl Zeiss, Jena, Germany). The method was validated with an accuracy of 92% using brown bread (BCR191, Sigma-Aldrich, St. Louis, MO, USA), a certified reference material. All diet samples were analyzed in triplicate.

Ca analysis in tissues

To determine Ca content in tissues, the samples were mineralized in a microwave digestion system (Speedwave Xpert, Berghof, Eningen, Germany) with pure nitric acid (Merck, Kenilworth, NJ, USA). After digestion, the samples were mixed with deionized water and then diluted with $LaCl_3$ (0.5%). The concentration of minerals was determined by flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany) at a wavelength of $\lambda = 422.7$ nm. The method was validated with an accuracy of 91% using bovine liver (1577 C, Sigma-Aldrich, St. Louis, MO, USA), a certified reference material.

Histological analysis

The resected femoral bones were fixed with 10% buffered formalin for 24 h. Then, the specimens were decalcified in Osteodec bone marrow biopsy decalcifying solution for another 3 h. Subsequently, each specimen was routinely processed and embedded separately in paraffin blocks. Two-micrometer-thick sections were cut from the blocks (three sections for each tissue sample) and stained with hematoxylin and eosin. Each slide contained two femoral bone sections with the bone marrow content. The bone marrow of each bone was analyzed under a light microscope (Leica, Allendale, NJ, USA), and the content of adipose tissue in the bone marrow was assessed separately by two scientists under a high-power field (HPF; 400× magnification). The percentile amount of fat bone marrow in the bone marrow was estimated under a light microscope in five different HPF areas (400× magnification, area of 0.25 mm^2), and the mean value was calculated. The number of osteoblasts, osteocytes, and osteoclasts was counted in each HPF area (400× magnification, area of 0.25 mm^2). The percentile amount of woven bone was also estimated under a light microscope in five different HPF areas (400× magnification, area of 0.25mm^2), and the mean percentile amount in the entire bone was calculated.

Biochemical parameters

The serum concentrations of PTH, PINP, OC, and ES were determined by ELISA using commercial ELISA kits (SunRed, Shanghai, China) which are used to estimate the mentioned parameters in samples of rat serum, blood, and plasma, and in other related tissue liquids. The precision of the technique used was validated, and the intra-assay and inter-assay precision (CV (%) = SD/mean × 100) for the estimation of ES, PTH, PINP, and OC was found to be < 9% and < 11%, respectively. The sensitivity of the method for each determined parameter was as follows: 3.112 ng/l for ES, 0.227 ng/dl for PTH, 0.325 ng/ml for PINP, and 0.523 ng/ml for OC. The analysis was carried out using an infinite F50 spectrometer (Tecan Group Ltd., Männedorf, Switzerland). The ELISA kits used were based on the principle of the dual antibody sandwich technique for the detection of parameters in rats' materials.

Statistical analysis

Statistical analysis was performed using the Statistica program (StatSoft, Tulsa, OK, USA). The normality of the distribution of the variables was determined using the Shapiro–Wilk test. Statistical differences between the analyzed groups were determined using a one-way analysis of variance with Tukey's post hoc test. P-value < 0.05 was considered statistically significant. The results are presented as mean values ± standard deviation.

Results

Table 1 shows the composition of the standard diet provided to groups C, OVX_C, and OVX_B and that of the diet with enriched pumpkin provided to groups OVX_P and OVX_P_B. The content of macronutrients and Ca was comparable in both diets.

Ovariectomy causes changes in body composition and in estrogen levels. In this study, ovariectomized rats showed a significant increase ($P < 0.05$) in body mass (Table 2). However, modified diets did not significantly ($P > 0.05$) affect the weight of rats in the ovariectomized groups (Table 2). As expected, ovariectomy also caused a significant decrease ($P < 0.05$) in serum ES concentration in rats (Table 3). An analysis of the parameters of

Table 1 Composition of diets (mean ± standard deviation)

Components	Diets	
	Standard (C/OVX_C/OVX_B groups)	Enriched pumpkin (OVX_P/OVX_P_B groups)
Caloric value (kcal/100 g)	326.37 ± 4.48	311.59 ± 3.29
Carbohydrates (g/100 g)	47.92 ± 0.60	41.6 ± 2.06
Fiber (g/100 g)	23.04 ± 0.60	23.92 ± 0.69
Fat (g/100 g)	3.76 ± 0.41	4.17 ± 0.38
Protein (g/100 g)	13.70 ± 0.21	14.95 ± 1.6
Ca (mg/g)	5.63 ± 0.37	5.57 ± 0.38

C control group, *OVX_C* ovariectomized group, *OVX_B* ovariectomized group receiving alendronate; *OVX_P* ovariectomized group receiving pumpkin enriched with $CaCO_3$, *OVX_P_B* ovariectomized group receiving pumpkin enriched with $CaCO_3$ and alendronate; alendronate concentration:3 mg/kg body weight

Table 2 Daily intake and body mass in rats (mean ± standard deviation)

Parameter	Group				
	C	OVX_C	OVX_P	OVX_B	OVX_P_B
Daily intake of diet (g)	24.98 ± 0.54	25.17 ± 1.49	23.74 ± 0.97	24.76 ± 0.28	24.19 ± 1.3
Daily intake of Ca (mg)	140.53 ± 3.05	141.61 ± 8.4	133.94 ± 3.05	142.39 ± 1.61	136.19 ± 7.31
Body mass (g)	338.59 ± 29.71[a]	442.71 ± 29.63[b]	430.31 ± 14.94[b]	439.01 ± 29.07[b]	424.05 ± 30.19[b]

C control group, *OVX_C* ovariectomized group, *OVX_P* ovariectomized group receiving pumpkin enriched with $CaCO_3$; *OVX_B* ovariectomized group receiving alendronate, *OVX_P_B* ovariectomized group receiving pumpkin enriched with $CaCO_3$ and alendronate; alendronate concentration:3 mg/kg body weight

[a,b]Significant differences between groups ($p < 0.05$)

Table 3 Level of estradiol and parameters of Ca metabolism in serum of rats (mean ± standard deviation)

Parameter	Group				
	C	OVX_C	OVX_P	OVX_B	OVX_P_B
ES (ng/l)	49.88 ± 5.06[b]	23.39 ± 5.55[a]	22.03 ± 5.27[a]	18.06 ± 2.65[a]	18.79 ± 3.59[a]
PINP (ng/ml)	3.55 ± 0.83[a]	4.6 ± 0.86[ab]	4.6 ± 0.5[ab]	4.58 ± 1.03[ab]	5.17 ± 1.03[b]
PTH (ng/dl)	3.44 ± 0.48[ab]	2.56 ± 0.48[a]	3.54 ± 0.71[b]	3.58 ± 0.74[b]	3.12 ± 0.42[ab]
OC (ng/ml)	18.57 ± 3.8	16.28 ± 1.17	17.56 ± 4.86	18.5 ± 6.05	16.39 ± 4.25

C control group, *OVX_C* ovariectomized group, *OVX_B* ovariectomized group receiving alendronate, *OVX_P* ovariectomized group receiving pumpkin enriched with $CaCO_3$, *OVX_P_B* ovariectomized group receiving pumpkin enriched with $CaCO_3$ and alendronate; alendronate concentration:3 mg/kg body weight

[a,b] significant differences between groups ($p < 0.05$)

Ca metabolism was also performed in this study, and the results are presented in Table 3. It was observed that ovariectomy had no significant effect ($P > 0.05$) on the concentration of PINP, while the combination of alendronate and enriched pumpkin caused a significant increase ($P < 0.05$) in the level of this bone formation marker in comparison to the control group. Similarly, ovariectomy did not significantly ($P > 0.05$) influence affect the PTH levels in the serum of rats. However, the addition of enriched pumpkin and alendronate alone in the diet caused an increase in PTH levels in rats in comparison to the OVX_C group, but this effect was not observed in rats that received the diet containing both these substances (OVX_P_B group).

To estimate the effect of modified diets on the Ca status in rats, the content of this element was estimated in the collected tissue samples and serum (Table 4). It was found that ovariectomy caused a significant reduction ($P < 0.05$) in Ca content in the femur. In turn, the addition of enriched pumpkin to the diet increased the femoral Ca concentration, and a similar effect was observed in the group that received the diet with alendronate. The addition of alendronate and enriched pumpkin (OVX_P_B group)

Table 4 Ca content in serum and tissues (mean ± standard deviation)

Parameter	Group				
	C	OVX_C	OVX_P	OVX_B	OVX_P_B
Serum (µg/ml)	132.65 ± 12.81	121.3 ± 8.76	112.94 ± 7.03	115.9 ± 8.48	124.43 ± 18.43
Femur (mg/g dm)	239.28 ± 18.01^{b}	217.22 ± 8.16^{a}	279.17 ± 12.83^{c}	290.94 ± 15.55^{c}	256.96 ± 10.22^{b}
Pancreas (µg/dm)	110.11 ± 12.19	114.2 ± 11.27	102.89 ± 18.82	97.79 ± 10.97	108.15 ± 11.98
Hair (µg/g dm)	603.68 ± 170.1^{b}	463.87 ± 55.87^{ab}	369.4 ± 78.73^{a}	413.12 ± 99.01^{a}	451.46 ± 54.8^{a}
Spleen (µg/g dm)	527.47 ± 112.62^{c}	460.31 ± 98.7^{c}	425 ± 54.04^{bc}	347.39 ± 27.01^{ab}	289.1 ± 33.07^{a}
Liver (µg/g dm)	156.01 ± 9.01^{bc}	140.74 ± 11.84^{bc}	94.91 ± 12.37^{a}	134.84 ± 21.87^{b}	159.54 ± 15.44^{c}
Heart (µ/g dm)	118.55 ± 16.79^{b}	83.9 ± 8.14^{a}	80.98 ± 7.47^{a}	81.5 ± 11.38^{a}	72.41 ± 8.67^{a}
Brain (µg/g dm)	178.93 ± 27.45	227.33 ± 85.69	219.14 ± 81.91	196.76 ± 86.43	239.07 ± 85.09
Muscle (µg/g dm)	47.4 ± 4.08^{c}	52.81 ± 11.46^{c}	42.9 ± 4.91^{bc}	34.37 ± 5.4^{ab}	25.25 ± 6.36^{a}
Kidney (µg/g dm)	91.19 ± 11.26^{a}	79.36 ± 8.19^{a}	148.66 ± 29.68^{b}	257.4 ± 42.85^{c}	461.07 ± 40.67^{d}

C control group, *OVX_C* ovariectomized group, *OVX_B* ovariectomized group receiving alendronate, *OVX_P* ovariectomized group receiving pumpkin enriched with $CaCO_3$, *OVX_P_B* ovariectomized group receiving pumpkin enriched with $CaCO_3$ and alendronate; alendronate concentration:3 mg/kg body weight; dm, dry mass

[a,b,c,d]Significant differences between groups ($p < 0.05$)

in combination also caused an increase in Ca concentration in the femur; however, this effect was less pronounced than that observed with the use of enriched pumpkin (OVX_P group) and alendronate (OVX_B group) alone. Ovariectomy caused a significant reduction ($P < 0.05$) in Ca content in the heart. The concentration of Ca in the spleen was significantly lower ($P < 0.05$) in the OVX_B and OVX_P_B groups compared to the OVX_C group. In the OVX_P group, a significant decrease ($P < 0.05$) in the Ca level was observed in the liver in comparison to the OVX_C group. Ovariectomy had no effect on Ca content in muscles, while the addition of alendronate alone and in combination with enriched pumpkin to the diet caused a significant reduction ($P < 0.05$) in the muscle Ca content. The use of modified diets led to a significant increase ($P < 0.05$) in the Ca content in the kidneys (almost twofold in the OVX_P group, threefold in the OVX_B group, and fivefold in the OVX_P-B group). The diet containing both alendronate and enriched pumpkin promoted more Ca accumulation in the kidneys than the diet containing either of these components.

To assess bone structure and bone health related to Ca metabolism, the study also included a histological analysis of the femur in rats (Table 5). The changes observed in the bone structure are presented in Figs. 2, 3, 4 and 5. It was found that ovariectomy did not affect the numbers of osteoblasts and osteocytes, but the addition of alendronate with or without enriched pumpkin to the diet caused a significant increase ($P < 0.05$) in these parameters in rats in comparison to the OVX_C group. Moreover, ovariectomy reduced the number of osteoclasts and increased fat bone marrow, but modified diets did not reverse this effect. Ovariectomy also caused an increase in the percentage of woven bone, but alendronate and enriched pumpkin, even when used alone, reversed this effect.

The study also analyzed the relationships between the examined parameters, and the results of the correlation analysis are presented in Table 6. A significant negative

Table 5 Parameters of the histological analysis of the femur (mean ± standard deviation)

Parameter	Group				
	C	OVX_C	OVX_P	OVX_B	OVX_P_B
Number of osteoblasts	10 ± 3.78^{a}	10.5 ± 5.13^{a}	16 ± 7.01^{ab}	20 ± 4.5^{b}	18.63 ± 5.93^{b}
Number of osteocytes	38.75 ± 8.35^{a}	45 ± 8.86^{ab}	40.25 ± 11.85^{a}	57.5 ± 8.02^{bc}	61.38 ± 11.99^{c}
Number of osteoclasts	0.88 ± 0.99	0 ± 0	0 ± 0	0.25 ± 0.46	0 ± 0
Bone marrow fat (%)	8.13 ± 3.72^{a}	43.75 ± 10.61^{b}	46.25 ± 9.16^{b}	36.25 ± 5.18^{b}	38.75 ± 6.41^{b}
Woven bone (%)	8.13 ± 2.59^{a}	18.75 ± 8.35^{c}	10 ± 4.63^{ab}	11.25 ± 3.54^{ab}	16.88 ± 3.72^{bc}

C control group, *OVX_C* ovariectomized group, *OVX_B* ovariectomized group receiving alendronate, *OVX_P* ovariectomized group receiving pumpkin enriched with $CaCO_3$, *OVX_P_B* ovariectomized group receiving pumpkin enriched with $CaCO_3$ and alendronate, alendronate concentration:3 mg/kg body weight

[a,b,c]Significant differences between groups ($p < 0.05$)

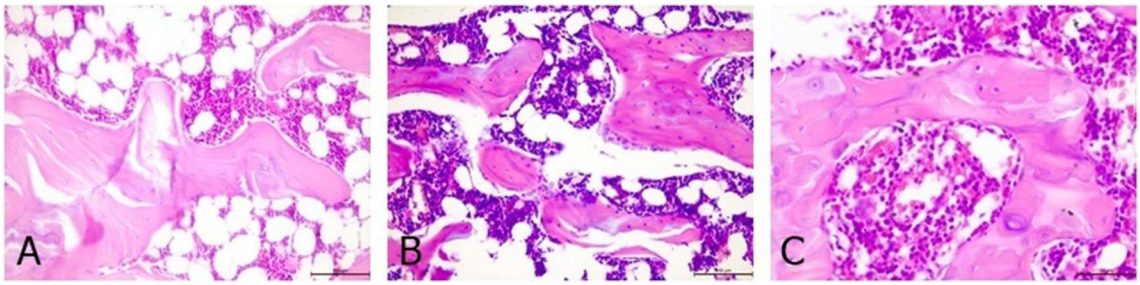

Fig. 2 Differences between the number of osteoblasts: **A** few osteoblasts along the bones in the representative of the OVX_C group (H&E; 100×); **B** numerous clusters of osteoblasts arranged along the bones in the representative of the OVX_B group (H&E; 100×); **C** numerous clusters of osteoblasts arranged along the bones in the representative of the OVX_P_B group (H&E; 100×)

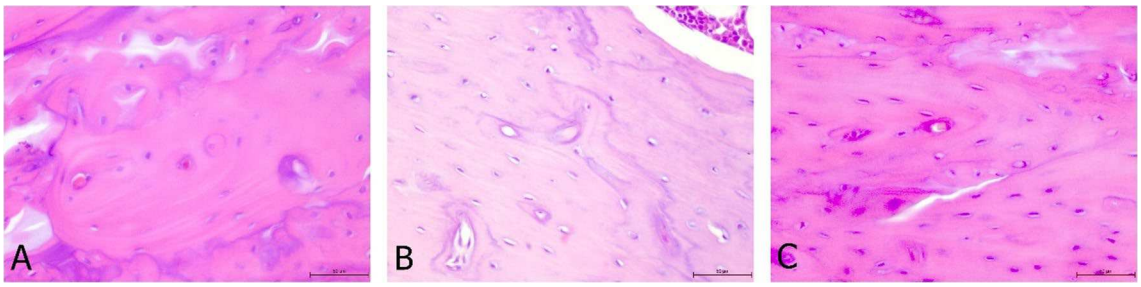

Fig. 3 Differences between the number of osteocytes: **A** few osteocytes in the bone in the representative of the C group (H&E; 200×); **B** average number of bone osteocytes in the representative of the OVX_C group (H&E; 200×); **C** large number of bone osteocytes in the representative of the OVX_B group (H&E; 200×)

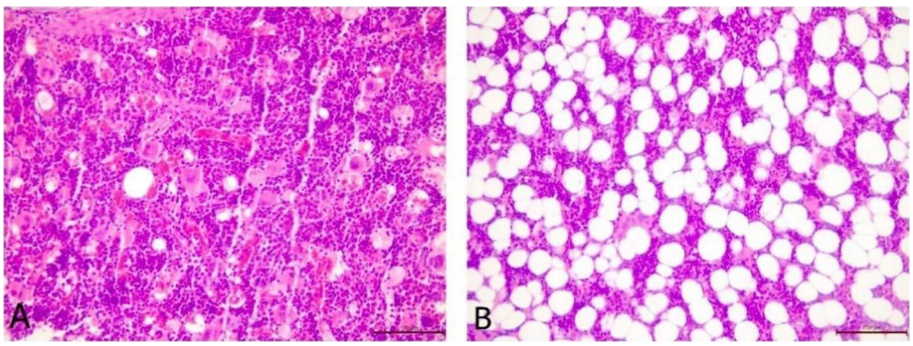

Fig. 4 Differences between the amount of bone marrow femoral adipocytes: **A** low number of bone marrow femoral adipocytes in the representative of the C group (H&E; 400×); **B** several number of bone marrow femoral adipocytes in the representative of the OVX_C group (H&E; 400×)

correlation ($P < 0.05$) was found between body mass and serum ES level ($r = -0.67$) as well as between body mass and serum Ca concentration ($r = -0.51$). Similarly, a negative correlation in Ca level was found between the kidney and the spleen ($r = -0.73$), between the kidney and muscle ($r = -0.93$), and between the femur and the pancreas ($r = -0.56$). A negative correlation was also found between the Ca content in muscles and the P1NP level in serum ($r = -0.62$). A positive correlation was observed between the PTH level in serum and the Ca level in the femur ($r = 0.64$).

Discussion

The results of the study showed that $CaCO_3$-enriched pumpkin increased bone Ca content to the same extent as alendronate. This is a valuable finding as it may indicate that Ca combination with pumpkin can prevent bone resorption and contribute to an increase in bone formation. Because the Ca content was comparable in the tested diets, some ingredients in pumpkin could have improved Ca bioavailability from enriched pumpkin, which contains large amounts of inulin, and Ca metabolism, which might affect bone structure. It has been shown that inulin can improve Ca bioavailability

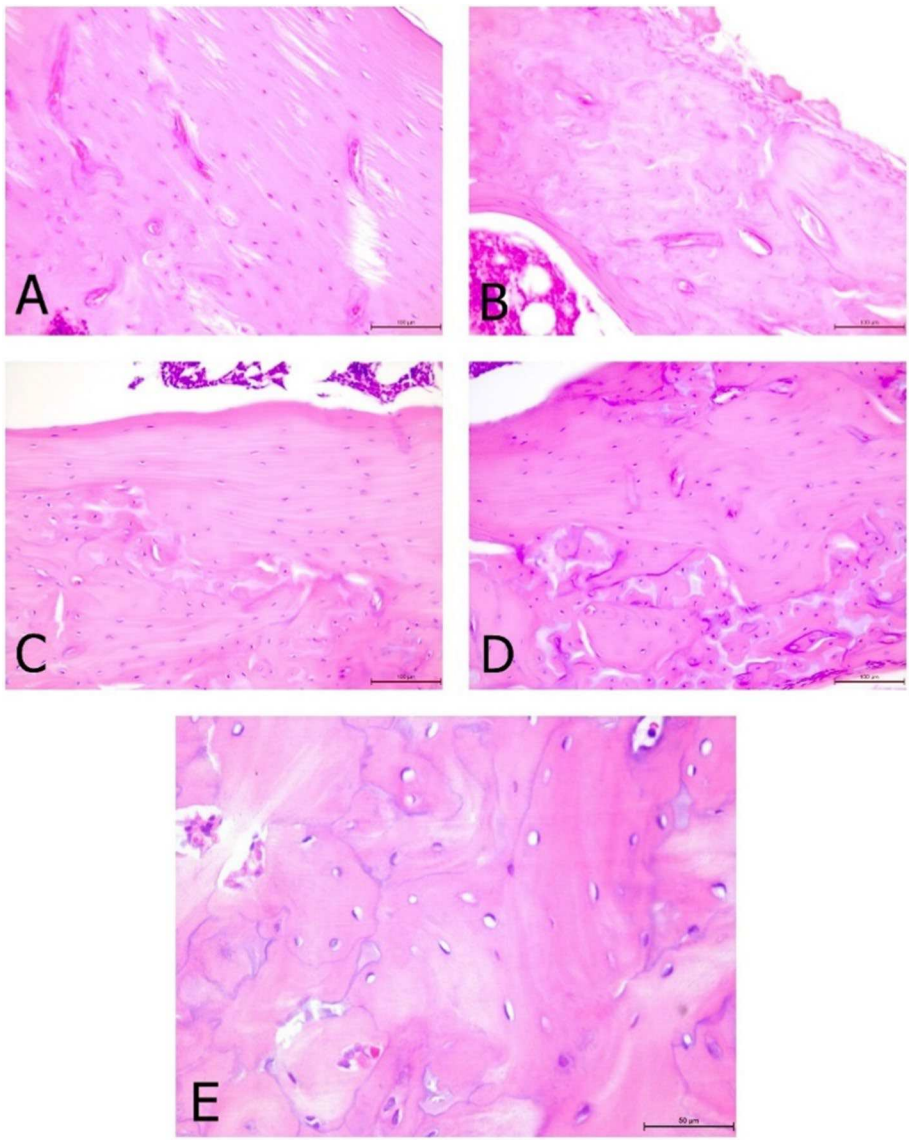

Fig. 5 Differences between the content of woven bones: **A** low content of woven bone in the representative of the C group (H&E; 200×); **B** high content of woven bone in the representative of the OVX_C group (H&E; 200×); **C** average content of woven bone in the representative of the OVX_P group (H&E; 200×); **D** average content of woven bone in the representative of the OVX_B group (H&E; 200×); **E** high content of woven bone in the representative of the OVX_P_B group (H&E; 400×)

Table 6 Significant ($P < 0.05$) Pearson correlation coefficient (r)

Parameters	r
Body mass (g) and ES (ng/l)	–0.67
Body mass (g) and Ca in serum (µg/ml)	–0.51
Ca in kidney (µg/g dm) and Ca in spleen (µg/g dm)	–0.73
Ca in kidney (µg/g dm) and Ca in muscle (µg/g dm)	–0.93
Ca in femur (mg/g dm) and Ca in pancreas (µg/g dm)	–0.56
PINP (ng/ml) and Ca in muscle (µg/g dm)	–0.62
PTH (ng/dl) and Ca in femur (mg/g dm)	0.64

dm, dry mass

in the intestine and can stimulate the transport of active Ca ions to cells, probably by increasing the level of calbindin (a transport protein) (Nzeusseu et al. 2006; Bakirhan and Karabudak 2021). Furthermore, from this study, it seems that Ca ions are shifted between tissues and that these ions may accumulate in bones at the expense of other tissues (Li et al. 2019; He et al. 2020). This mechanism was partly confirmed by the inverse correlation observed between Ca content in the pancreas and femur. Moreover, pumpkin contains other ingredients such as carotenoids, zeaxanthin, lutein, which could prevent bone resorption in rats after ovariectomy (Yamaguchi 2012; Ozaki et al. 2015; Takeda et al. 2017; Tominari et al. 2017). However, the changes observed in Ca content in the femur are difficult to associate with PTH levels. Although the levels of PTH were not changed by ovariectomy, the addition of alendronate and enriched pumpkin to the diet contributed to an increase in this hormone. The results obtained for PTH concentration in ovariectomized groups were unexpected. It is challenging to directly explain the low PTH concentration observed in the OVX_C group and the relatively high concentrations observed in the

OVX_P and OVX_B groups since the opposite relationship was expected. Additionally, it was surprising to find a positive correlation between femoral Ca concentrations and PTH concentrations. It appears that in the pumpkin and alendronate groups, the observed relationships are associated with the high accumulation of Ca in the kidneys. PTH stimulates Ca reabsorption in the kidneys and promotes its accumulation. The observed relationships undoubtedly have a multidirectional aspect. In ovariectomized rats with low estrogen levels, we expected adverse bone changes, but these rats were not Ca-deficient, and all diets had adequate amounts of Ca. As a result of changes in bones, we observed an increase in PTH, which influenced the kidneys by inhibiting Ca excretion, and Ca possibly was delivered to the bones via the action of other factors, such as ion shifts between tissues, or by biologically active substances of the drug or pumpkin components. Research suggests that the action of lycopene and carotenoids on bones is related to the activity of PTH (Burri et al. 2016). Moreover, obesity and increased bone marrow fat in the bones observed in ovariectomized rats might have an influence on the noticed changes in biochemical parameters. Other studies have shown a correlation between obesity and bone marrow fat and PTH activity (Rao et al. 2003; Fan et al. 2017). Weight gain in rats with low estrogen levels was expected, as was the increase in bone marrow fat, and this may possibly affect PTH levels in rats (Guasch et al. 2012), hence the lack of expected relationships between ovariectomy, bone Ca, and PTH. Unexpectedly, we did not observe significant changes ($P > 0.05$) in PINP and OC levels in ovariectomized groups. PINP and OC are nonspecific collagen proteins, mainly produced by osteoblasts, and their content in the blood can reflect the activity of osteoblasts (Guo et al. 2021). Although the number of osteoblasts increased in groups fed with diets containing pumpkin and alendronate alone and in combination, the relative increase in PINP level was only observed in OVX_P_B group, which may indicate an increased intensity of bone turnover due to the presence of two factors: bioactive ingredients of enriched pumpkin and alendronate. We also observed a link between the PINP level and changes in Ca in the body, as evidenced by the negative correlation between the PINP level and muscle Ca content. In the intervention groups after ovariectomy, Ca from the muscles was probably shifted to the bones and to the kidneys, as indicated by significant correlations ($P < 0.05$) between Ca content in these organs. Moreover, in ovariectomized rats, a significant decrease ($P < 0.05$) in Ca in the heart was observed, which might lead to problems with myocardial contractility. Other studies have confirmed that after ovariectomy, the sensitivity of Ca^{2+} myofilament is reduced, which leads to the release of Ca ions from the heart (Fares et al. 2013). An unexpected finding of this study is that the use of modified diets resulted in Ca accumulation in the kidneys. Unfortunately, no parameters of kidney functioning were analyzed, and histological analysis of the kidneys was not performed in this study. However, it can be assumed that Ca ions from other tissues were transported to the kidneys in the rats that received modified diets. In a study by Nijenhuis et al., a significant increase ($P < 0.05$) in the expression of TRPV5 (a protein responsible for the transport of Ca ions) was observed in bones following the administration of alendronate, while no such increase was observed in the kidneys and intestine (Nijenhuis et al. 2008). On the other hand, alendronate has been known to cause damage to the kidneys by forming Ca aggregates, which can lead to the formation of kidney stones or glomerulonephritis (Song and Maalouf 2000). Because the kidneys are responsible for the reabsorption of Ca, stones can restrict their filtration, resulting in hypercalciuria, and consequently, a decrease in Ca concentration in the blood (Han et al. 2019). Enriched pumpkin contains ingredients that can affect kidney functioning. Inulin, which is one such ingredient, can expose the kidneys to a high amount of floating Ca due to its ability to increase Ca excretion (Adolphi et al. 2009). Large amounts of vitamins A and E found in pumpkins can lead to glomerular hyperfiltration and ultimately affect the filtration ability of the kidneys (Kedishvili 2016; Parente Filho et al. 2020; Chen et al. 2021).

For a detailed interpretation of the results, it is also worth paying attention to the results of histological analysis. In this study, the histopathological analysis of the femurs revealed interesting facts regarding bone cells, fat bone marrow degeneration, and woven bone. Osteoblasts are bone cells formed from mesenchymal precursors and eventually differentiate into osteocytes. Both osteoblasts and adipocytes are derived from the same stem cells, and thus a large amount of adipose tissue is an indicator of a large number of osteoblasts (Kos-Kudła et al. 2019). In this study, we observed a high number of both these cell types in ovariectomized rats; however, the increase in these cells was statistically significant ($P < 0.05$) only in the groups that received alendronate-supplemented diet, which suggests that stimulation of osteoblast differentiation intensifies the bone-building process (Ma et al. 2018). Rats with a high amount of adipose tissue also have a high percentage of adipose tissue marrow, as has been confirmed by previous studies on humans (Horowitz et al. 2017; van der Eerden and van Wijnen 2017) and animals (Iwaniec and Turner 2013; Fan et al. 2015). An interesting observation from these studies is the increased percentage of woven bone (immature bone) in the remodeling phase (Shapiro and Wu 2019). Woven bone is formed very quickly and appears porous. The proportion of woven bone is generally high during growth and puberty. On the other hand, in adults, this bone constitutes about 5–10%, while its higher share indicates structural overload or trauma, which is a temporary effect associated

with the reconstruction of the lamellar (mature) bone (Hart et al. 2020). In this study, we observed that ovariectomy caused a significant increase ($P < 0.05$) in the percentage of woven bone, while the addition of enriched pumpkin and alendronate to the diet resulted in an opposite effect. Thus, it can be concluded that the reduction in estrogen levels led to the need for bone reconstruction in ovariectomized rats, as indicated by the increase in the percentage of woven bone in these animals. On the other hand, inulin and $CaCO_3$ present in enriched pumpkin and alendronate accelerated bone reconstruction by increasing bone formation (discussed earlier), thus reducing the share of woven bone.

Limitations

Due to several limitations of this study, some of the obtained results could not be highlighted here. Because rats' urine was not collected in the study, we could not state whether its excretion increased with the accumulation of Ca in the kidneys. Other parameters related to bone metabolism, such as vitamins K and D, were not analyzed because only limited volume of serum was obtained from rats. The study also did not include a histological analysis of the kidneys, which could have been helpful in explaining the mechanism of Ca accumulation in this tissue. Furthermore, the study did not have a sham-operated control, and therefore the effect of sham surgery on rats was not analyzed; however, the results obtained in the ovariectomized group were compared with the nonoperated control group and the ovariectomized group fed with a standard diet. Unfortunately, we did not study the group with unenriched pumpkin, so we cannot determine what changes would occur in the rats' organism if the pumpkin was not subjected to osmotic dehydration.

Conclusion

CaCO3-enriched pumpkin can improve the concentration of Ca in the femur and bone recovery in ovariectomized rats, which is similar to the effect of alendronate. However, enriched pumpkin causes Ca accumulation in the kidneys, which is exacerbated when it is used in combination with alendronate. Further research is needed to elucidate the mechanism of calcium accumulation in the kidneys as a result of the consumption of calcium carbonate-enriched pumpkins.

Authors' contribution Conceptualization, NW, JS: methodology, JS, NW, PK: (PK); software, JS, NW: validation, NW, JS: formal analysis, JS: investigation, NW, PK (PK), JS; resources, AGM: data curation, JS, NW: writing—original draft preparation, NW, JS: writing—review and editing, all authors; visualization, NW, JS: supervision, JS: project administration, AGM: funding acquisition, AGM. All authors have read and agreed to the published version of the manuscript.

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Data availability The data used to support the findings of this study can be made available by the corresponding author upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have declared that they have no conflict of interest.

Ethics approval All experimental procedures were performed in accordance with the EU Directive 2010/63/EU for animal experiments. Approval for the study was obtained from the Local Ethics Committee in Poznań (no. 34/2019). The reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Consent to participate Not applicable.

Consent for publication Not applicable.

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EFFECTS OF OVARIECTOMY AND CALCIUM ENRICHED PUMPKIN ON MAGNESIUM STATUS IN RATS

Natalia Wawrzyniak[1], Anna Gramza-Michałowska[2], Joanna Suliburska[1]✉

[1]Department of Human Nutrition and Dietetics, University of Life Sciences
Wojska Polskiego 31, 60-624 Poznań, **Poland**
[2]Department of Gastronomy Sciences and Functional Foods, University of Life Sciences
Wojska Polskiego 31, 60-624 Poznań, **Poland**

ABSTRACT

Background. Calcium (Ca) and magnesium (Mg) are important components of bones, whose homeostasis is disturbed during menopause. Calcium and magnesium metabolism are closely related, so it is important to study the interactions between them. This study aimed to determine the effect of Ca-enriched pumpkin on the Mg content in tissues in an animal model of postmenopausal osteoporosis.

Material and methods. 70 female Wistar rats divided into seven groups. One group was fed a standard diet (C), whereas the other six groups were ovariectomized and fed a standard diet (OVX), a calcium-deficient diet (DEF), a calcium lactate diet (CaL), calcium-lactate-enriched pumpkin (P_CaL), calcium lactate and alendronate (CaL_B), or calcium-lactate-enriched pumpkin and alendronate (P_CaL_B). This nutritional intervention was followed for 12 weeks, and then the rats were euthanized. Tissue samples were collected, and their magnesium content was assessed.

Results. The Mg content in bones was lower in the OVX group (3.15 ±0.19 mg/g) but higher in the DEF group (3.76 ±0.16 mg/g) in comparison with the control group (3.45 ±0.15 mg/g). The Mg content in the muscles and the liver was higher in the P_CaL group (1025.24 ±47.22 μg/g and 2102.09 ±83.35 μg/g) compared with the control group (842.51 ±19.13 μg/g and 1486.15 ±97.12 μg/g). However, the CaL_B and P_CaL_B groups showed a high Mg content in the kidneys (about 156% of the control group).

Conclusion. Ovariectomy and intervention diets revealed various new observations regarding the effect of innovative calcium-rich foods on the Mg content. These results showed that (i) ovariectomy decreases the status of Mg content; (ii) deficiency of Ca in the diet and Ca-enriched pumpkin with alendronate improve the Mg content in bones; and (iii) alendronate promotes the accumulation of Mg in the kidneys. In postmenopausal women, both those treated and untreated with drugs and diet, magnesium status should be monitored.

Keywords: ovariectomy, calcium, magnesium, postmenopausal osteoporosis, ovariectomized rats

ABBREVIATIONS

Ca – calcium
Mg – magnesium
BMD – bone mineral density
AAS – flame atomic absorption spectrometry
TRPM6 – transient receptor potential cation channel subfamily M member 6

INTRODUCTION

Menopause begins in women around the age of 45–50. Clinicians define menopause as the last menstruation in women's lives, and this condition is associated with various changes in metabolism. The primary symptom of menopause is a decline in estrogen levels due to the suppression of endogenous ovarian function (Minkin,

✉joanna.suliburska@up.poznan.pl, https://orcid.org/0000-0002-0937-8427

2019). A low estrogen concentration in the blood has numerous consequences, including a reduction in bone mineral density (BMD), resulting in postmenopausal osteoporosis (Ji and Yu, 2015). Deepening low BMD leads to an increase in bone fragility, and thus an increase in the risk of falls and bone fractures (Miller, 2016). The effects of osteoporosis significantly reduce the quality of life of patients and their families, which is why it is important to prevent and effectively treat this disease (Erhan and Ataker, 2020). Osteoporosis is treated using either pharmacological measures, e.g., the use of alendronate or denosumab, or nonpharmacological methods, which include, among others, an adequate calcium (Ca) intake.

Ca is the primary bone mineral; therefore, its adequate supply to the body is an important preventive factor for osteoporosis. It is present in bones in the form of hydroxyapatite [$(Ca)_{10}(PO_4)_6(OH)_2$], and about 99% of the total Ca in the body is present in bones (Murshed, 2018). Bone reconstruction, i.e., the replacement of the tissue with a new one, occurs throughout life; however, during menopause, bone resorption dominates over bone formation, which leads to a significant reduction in BMD (Song, 2017). Bone changes occurring during osteoporosis depend on Ca metabolism, and an adequate Ca supply supports the treatment of osteoporosis and increases BMD, thus preventing fractures (Black and Rosen, 2016).

The standard recommendation level of Ca for women during menopause is between 1000 and 1500 mg per day (Black and Rosen, 2016). In many countries, Ca is deficient in the diet, and its daily intake ranges from around 400 mg in Asia to around 700 mg in Africa. Only in the Scandinavian countries is Ca intake around 1200 mg/day (Balk et al., 2017). In Poland, calcium is also not consumed in the right dose (about 700 mg per day) (Skowrońska-Jóźwiak et al., 2016) and according to the Institute of Food and Nutrition, the recommended daily intake for women over 50 is 1200 mg (Jarosz et al., 2020). Ca supplements and Ca-fortified foods can help maintain normal blood Ca levels. Supplements should be used as prescribed by the doctor since frequent excessive dosage leads to side effects such as gastrointestinal complaints and the formation of kidney stones (Chiodini and Bolland, 2018). Ca can be supplemented in the diet using enriched or fortified foods (Harvey and Diug, 2018). Effective saturation of plant tissues with Ca can enrich the diet, thus contributing to an increase in Ca consumption and to the prevention and treatment of osteoporosis (Kulczyński et al., 2021). Osmotic dehydration makes pumpkin tissues saturated with calcium along with the active substance – inulin, which increases the bioavailability of calcium (Krupa-Kozak et al., 2016). The aim of producing pumpkin enriched with calcium was to create a food product, which would be a good source of calcium (Wawrzyniak et al., 2020; Wawrzyniak and Sulibursk a, 2021). However, excessive calcium levels can interfere with the metabolism of other minerals, including magnesium (Mg), due to numerous interactions (Perales et al., 2006; Pérez-Gallardo et al., 2009).

Ca metabolism and Mg metabolism are related in many ways. Ca absorption is dependent on the level of vitamin D in the body, and Mg is involved in the hydroxylation of vitamin D to its active form, $1,25(OH)_2D$, in the kidneys (Rosanoff et al., 2016). In this way, Mg plays an important role in Ca absorption. Even vitamin-D-resistant rickets becomes sensitive to calcitriol again under Mg supplementation (Wesselink et al., 2020). On the other hand, 1,25(OH)2D coordinates the intestinal absorption of Mg, which is related to Ca. Ca deficiency in the diet leads to a high turnover of vitamin D metabolism products and thus a lower vitamin D level (Lips, 2012). Mg deficiency results in an impaired parathyroid hormone (PTH) response (Uwitonze and Razzaque, 2018), and it is well known that PTH is involved in Ca metabolism. In Ca deficiency, PTH is secreted by the parathyroid cells (Goltzman et al., 2018). In addition, Mg influences the active transport of Ca ions through the cell membrane, which is crucial in muscle contraction, conduction of nerve impulses, normal heart rhythm, and vasomotor tension (Gröber et al., 2015).

Since Mg interacts with Ca and supplementation of these two minerals is positively correlated with BMD in postmenopausal women (Mahdavi-Roshan et al., 2015; Mutlu et al., 2007), it is interesting to investigate the effects of the consumption of innovative Ca-rich foods on Mg metabolism. Therefore, this study aimed to determine the effects of Ca-enriched pumpkin on the Mg content tissues in ovariectomized rats while the hypothesis of the study is that pumpkin enriched with Ca affects the status of Mg in ovariectomized rats.

METHODS

Experimental protocols

The rats were fed the standard AIN-93M diet with or without modification during the experiment (Reeves and Suppl, 1997). They were divided into seven groups of 10 rats each. No significant differences in the initial body weight were observed between the rats. Six groups (60 rats) were ovariectomized and subjected to a recovery period. Then, a 12-week nutritional intervention was introduced. The control group (C) and one of the ovariectomized groups (OVX) received the unmodified standard AIN-93M diet, whereas the other five groups received a modified diet: Ca-deficient diet (DEF), calcium lactate diet (CaL), pumpkin enriched with calcium lactate (P_CaL), alendronate and calcium lactate (CaL_B), or calcium-lactate-enriched pumpkin and alendronate (P_CaL_B). The experiment design is presented in Fig. 1, and the dietary components are summarized in the previous study (Wawrzyniak et al., 2022). Calcium lactate and enriched pumpkin were used in such an amount that the diets did not differ in the calcium content. The alendronate dose was set at 3 mg per kg body weight and adjusted weekly.

The rats were provided food and deionized water ad libitum, and intake was recorded daily. Their body weights were measured weekly, and a Bruker LF90II body composition analyzer was used for the analysis at the end of the experiment. The rats were euthanized by guillotine head removal. Femurs, pancreas, spleen, liver, heart, brain, muscles, and kidneys were isolated for analysis. Tissues were frozen at −80°C after washing with saline and weighing. Hair was collected from all rats from the interscapular area.

Materials and reagents

A pumpkin (yellow melon, *Cucurbita maxima*) obtained from an organic farm with the consent of the land owner was used in this study. Inulin and calcium lactate were purchased from Agnex (Białystok, Poland). Minerals and vitamins used for diet preparation were purchased from Sigma-Aldrich (Darmstadt, Germany). Casein, corn starch, dextrin, rapeseed oil, and sucrose were purchased from Hortimex (Konin, Poland).

Osmotic dehydration

The pumpkin tissue was enriched with calcium lactate in the process of osmotic dehydration as follows: the pumpkin flesh was cut into 1-cm^3 cubes and frozen. A solution of inulin and distilled water (50:50) was prepared in jars into which calcium lactate was added until a concentration of 5% was achieved. The frozen pumpkin cubes were added to the solution in a ratio of 1:5, and the mixture was shaken for 2 h in a 50°C

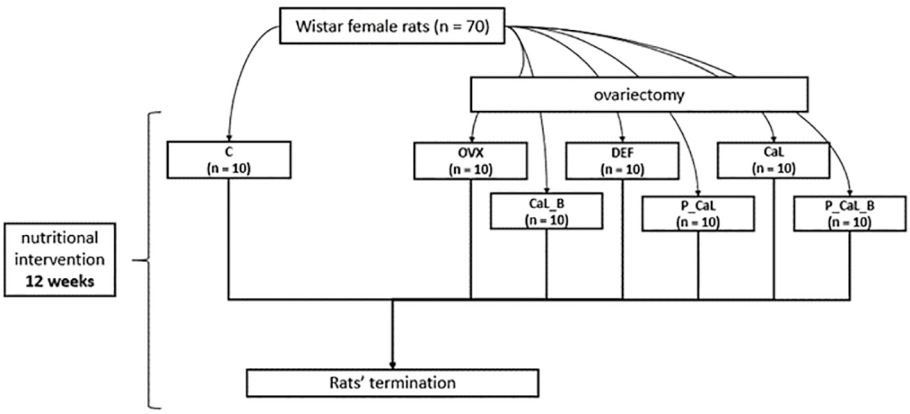

Fig. 1. Scheme of the study; C – control group; OVX – ovariectomized group; DEF – ovariectomized group with calcium-deficit diet; CaL – ovariectomized group with calcium lactate; CaL_B – ovariectomized group with calcium lactate and alendronate; P_CaL – ovariectomized group with calcium-lactate-enriched pumpkin enriched with; P_CaL_B – ovariectomized group with calcium-lactate-enriched pumpkin and alendronate

water bath, freeze-dried, ground, and added as such to the rats' diets (Wawrzyniak et al., 2022).

Animals

A total of 70 female Wistar rats aged 12 weeks were purchased from the Greater Poland Center for Advanced Technologies, University of Adam Mickiewicz in Poznań, Poland. All experimental procedures were performed in accordance with the EU Directive 2010/63/EU for animal experiments. Approval for the study was obtained from the Local Ethics Committee in Poznań (no. 34/2019). The reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Mg and Ca analysis in diets

To determine the Mg and Ca content in the diets, 1 g of each diet was burned in a muffle furnace at 450°C until mineralization. Then, the samples were dissolved in 1 mol/l nitric acid (Merck, Kenilworth, NJ, USA). Their mineral content was determined after diluting them with appropriate amounts of $LaCl_3$ (0.5%) and deionized water (AAS-3, Carl Zeiss, Jena, Germany) using flame atomic absorption spectrometry (AAS). A certified reference material was used to validate the method, with a 92% for Ca and 95% for Mg accuracy using brown bread (BCR191, Sigma-Aldrich, St. Louis, MO, USA). All samples were analyzed in triplicate.

Mg analysis in tissues

To determine the Mg content in tissues, the samples with pure nitric acid (Merck, Kenilworth, NJ, USA) were subjected to mineralization in a microwave digestion system (Speedwave Xpert, Berghof, Eningen, Germany). After digestion, they were diluted with deionized water and $LaCl_3$ (0.5%). The flame AAS method was used to determine the content of Mg (AAS-3, Carl Zeiss, Jena, Germany). A certified reference material — bovine liver — was used (1577C, Sigma-Aldrich, St. Louis, MO, USA) to validate the method (with an accuracy of 97%).

Statistical analysis

The Statistica program was used to conduct statistical analyses (StatSoft, Tulsa, OK, USA). To determine the normality of the distribution of the variables, the Shapiro–Wilk test was used. One-way analysis of variance with Tukey's post hoc test was used to identify the statistical differences between the analyzed groups (a p-value of <0.05 was considered statistically significant). For comparing two groups, Student's t-test was used. The results are presented in tables as mean values ± standard deviation.

RESULTS

The results are presented in Tables 1–3. The Mg content did not differ between the diets (Table 1). Similarly, no differences in the daily consumption of Mg were observed between the groups.

Ovariectomy-induced changes that were or were not compensated for by nutritional intervention were observed (Table 2). Ovariectomy significantly reduced the Mg content in bones, whereas the Ca-deficient diet and calcium lactate or enriched pumpkin (with and without alendronate) significantly increased it, compared with the C and OVX groups. Ovariectomy also led to a reduction in the Mg content in hair, but the dietary intervention did not result in any changes, except for the calcium lactate and alendronate group (CaL_B), whose Mg content was comparable to that of the control group. Although ovariectomy and a Ca-deficient diet did not affect the Mg content in the spleen, liver, heart, brain, and kidneys, significant changes in it were observed after the nutritional intervention. Calcium-lactate-enriched pumpkin significantly reduced the Mg content in the spleen (P_CaL), which was intensified by the addition of alendronate, resulting in a two-fold decrease in the Mg content in the spleen (CaL_B and P_CaL_B), compared with the OVX and control groups. Calcium lactate increased the Mg content in the liver in comparison with the control and ovariectomized group (OVX), as did enriched pumpkin, and alendronate exacerbated this effect. Both calcium lactate and enriched pumpkin decreased the Mg content in the heart, with the addition of alendronate bringing it closer to that of the control group. The modified diets did not significantly affect the Mg content in the brain in comparison with the OVX group, whereas in the kidneys, alendronate significantly increased it in the CaL_B and P_CaL_B groups, compared with the CaL and P_CaL groups. Ovariectomy increased the Mg content in muscles in comparison with the control group, which was not affected by the nutritional intervention.

Table 1. Diet parameters and body weight of rats (mean and standard deviation)

Parameter	Group						
	C	OVX	DEF	CaL	CaL_B	P_CaL	P_CaL_B
Ca content in diet, mg/g	5.63 ±0.37[b]	5.63 ±0.37[b]	0.64 ±0.04[a]	5.68 ±0.24[b]	5.68 ±0.24[b]	5.77 ±0.15[b]	5.77 ±0.15[b]
Mg content in diet, mg/g	0.57 ±0.06	0.57 ±0.06	0.55 ±0.03	0.58 ±0.03	0.58 ±0.03	0.58 ±0.01	0.58 ±0.01
Daily intake of diet, g	25.08 ±0.63	25.11 ±1.70	26.14 ±1.87	25.90 ±0.55	25.66 ±2.29	24.31 ±1.26	24.84 ±2.29
Daily intake of Ca, mg	141.12 ±3.56[b]	141.30 ±9.57[b]	16.77 ±1.20[a]	147.03 ±3.11[b]	139.73 ±12.44[b]	140.31 ±7.26[b]	145.01 ±13.39[b]
Daily intake of Mg, mg	14.30 ±0.36	14.31 ±0.97	14.59 ±1.20	15.23 ±0.51	15.19 ±1.31	14.01 ±0.73	14.50 ±1.56
Body weight g	325.86 ±25.97[a]	421.90 ±55.10[b]	441.00 ±70.97[b]	428.40 ±51.1[b]	433.30 ±51.62[b]	384.11 ±34.02[a,b]	392.30 ±34.88[a,b]

C – control group; OVX – ovariectomized group; DEF – ovariectomized group with calcium-deficit diet; CaL – ovariectomized group with calcium lactate; CaL_B – ovariectomized group with calcium lactate and alendronate; P_CaL – ovariectomized group with calcium-lactate-enriched pumpkin; P_CaL_B – ovariectomized group with calcium-lactate-enriched pumpkin and alendronate; Mg – magnesium.
a, b – significant differences between groups ($p < 0.05$).

Table 2. Magnesium content in the tissues (mean and standard deviation)

Tissue	Group						
	C	OVX	DEF	CaL	CaL_B	P_CaL	P_CaL_B
Femur mg/g dm	3.45 ±0.15[b]	3.15 ±0.19[a]	3.76 ±0.16[c]	3.86 ±0.16[c,d]	3.76 ±0.22[c]	3.88 ±0.19[c,d]	4.05 ±0.14[d]
Pancreas µg/g dm	118.78 ±8.01	109.48 ±8.28	116.51 ±17.38	109.63 ±12.11	112.74 ±9.19	116.38 ±6.18	114.91 ±9.03
Hair µg/g dm	94.79 ±14.38[b]	76.31 ±7.73[a]	78.29 ±6.25[a]	79.04 ±6.99[a]	83.62 ±6.65[a,b]	76.07 ±7.75[a]	82.04 ±4.77[a]
Spleen µg/g dm	1 874.87 ±416.66[c]	1 747.16 ±347.97[c]	1 462.65 ±340.31[b,c]	1 499.35 ±282.13[b,c]	885.92 ±235[a]	1 140.94 ±280.59[a,b]	902.89 ±214.49[a]
Liver µg/g dm	1 486.15 ±97.12[a]	1 402.83 ±112.88[a]	1 401.7 ±142.17[a]	1 870.98 ±142.55[b]	1 948.09 ±140.85[b,c]	2 102.09 ±83.35[c]	2 022.28 ±124.38[b,c]
Heart µg/g dm	1 122.79 ±40.57[c,d]	1 148.86 ±45.68[d]	1 119.4 ±46.74[c,d]	1 031.4 ±41.94[a,b]	1 063.51 ±55.62[b,c]	995.08 ±52.39[a]	1 063.59 ±50.81[a,b,c]
Brain µg/g dm	589.12 ±15.2[b]	568.36 ±26.31[a,b]	558.82 ±12.63[a]	557.18 ±12.06[a]	562.31 ±10.28[a]	569.59 ±13.54[a,b]	568.35 ±13.62[a,b]
Muscle µg/g dm	842.51 ±19.13[a]	994.13 ±44.25[b,c]	970.37 ±36.8[b,c]	952.08 ±40.79[b]	1 020.71 ±54.28[c]	1 025.24 ±47.22[c]	955.68 ±57.28[b]
Kidney µg/g dm	1 041.53 ±24.34[a]	1 036.33 ±64.53[a]	1 048.7 ±66.6[a]	1 052.25 ±61.27[a]	1 623.48 ±107.92[b]	1 054.13 ±83.14[a]	1 631.18 ±116.71[b]

C – control group; OVX – ovariectomized group; DEF – ovariectomized group with calcium-deficit diet; CaL – ovariectomized group with calcium lactate; CaL_B – ovariectomized group with calcium lactate and alendronate; P_CaL– ovariectomized group with calcium-lactate-enriched pumpkin; P_CaL_B – ovariectomized group with calcium-lactate-enriched pumpkin and alendronate; dm – dry mass.
a, b, c, d – significant differences between groups ($p < 0.05$).

Table 3. Significant changes in the magnesium content in tissues

Tissue	Ovariectomy	Ca deficit	Enriched pumpkin	Bisphosphonate	Bisphosphonate + enriched pumpkin
Bone	↓	↑			↑
Pancreas	↓				
Hair	↓				
Spleen			↓	↓	↓
Liver			↑		↑
Heart					
Brain			↑		
Muscle	↑		↑	↑	
Kidney				↑	↑

Compared groups: ovariectomy – C:OVX; Ca deficit – OVX:DEF; enriched pumpkin – CaL:P_CaL; bisphosphonate – CaL:CaL_B; bisphosphonate + enriched pumpkin – CaL:P_CaL_B.
C – control group; OVX – ovariectomized group; DEF – ovariectomized group with a calcium-deficit diet; CaL – ovariectomized group with calcium lactate; CaL_B – ovariectomized group with calcium lactate and alendronate; P_CaL – ovariectomized group with calcium-lactate-enriched pumpkin; P_CaL_B – ovariectomized group with calcium-lactate-enriched pumpkin and alendronate.

Significant changes in the Mg content in tissues are presented in Table 3. The effect of ovariectomy was investigated by comparing the control and ovariectomized groups (C:OVX). Moreover, the effect of Ca deficiency in the diet was evaluated by comparing the ovariectomized and Ca-deficient groups (OVX:DEF). The influence of Ca-enriched pumpkin on the Mg content was investigated by comparing the calcium lactate group and the calcium-lactate-enriched pumpkin group (CL:P_CaL). The effect of alendronate was determined by comparing the calcium lactate group and the calcium lactate and alendronate group (CaL:CaL_B), and the effect of combinations of enriched pumpkin with the drug was analyzed by comparing the enriched pumpkin group and the enriched pumpkin and alendronate group (CaL:P_CaL_B).

Ovariectomy reduced the Mg content in bones, pancreas, and hair but increased the same in muscles, compared with the control group. The Ca-deficient diet increased the Mg content in bones, compared with the ovariectomized group (OVX). The enriched pumpkin group (P_CaL) showed a higher Mg content in the liver, brain, and muscles but a lower Mg content in the spleen in comparison with the calcium lactate group (CaL). Bisphosphonates increased the Mg content in muscles and the kidneys but decreased it in the spleen, compared with the CaL group. The combination of pumpkin and bisphosphonate increased the Mg content in the bones, liver, and heart but decreased it in the spleen in comparison with the CaL group.

DISCUSSION

The results of this study showed that ovariectomy decreased the Mg content in the femur, whereas the Ca-deficit diet and Ca-enriched pumpkin improved the same in bones. Moreover, alendronate enhanced the Mg content in the kidneys.

It is well established that estrogen deficiency during menopause leads to various physiological and molecular changes (Wall et al., 2014). The disturbance in the Mg content in ovariectomy may be attributable to the decline in estrogen levels, which leads to dysregulation of Mg homeostasis by decreasing the intestinal absorption and reabsorption in the kidneys due to reduced activity and transcription of TRPM6 (transient receptor potential cation channel subfamily M member 6) — a channel for Mg^{2+} ions (Cao et al., 2009). Mg deficiency leads to the inhibition of bone growth, an increase in the number of osteoclasts, a decrease in the number of osteoblasts, and thinning of the bone trabeculae (Rude et al., 2003); therefore, a decrease in BMD is possible in the OVX group. The Mg content in hair and serum is closely related to BMD (Song et al., 2007); therefore, in the ovariectomized rats (OVX group), a decrease in the Mg content was observed in both bones and hair. However, a slightly lower Ca content in bones and a lower number of osteoblasts were observed in the previous study on ovariectomized rats, which may show the relationship between Ca and Mg, and bone structure during the development of adverse bone changes as a result of menopause, which is a long-term process (Wawrzyniak et al., 2021). The limitation of this study is that the serum Mg content was not determined, which

would have allowed for a broader explanation of the obtained results. As far as clinical trials are concerned, Laires et al. observed a low Mg content in red blood cells in healthy menopausal women, which indicates the dysregulation of factors that control Mg homeostasis during menopause (Laires et al., 2004). It has been reported that the serum Mg content in women with postmenopausal osteoporosis is much lower than the recommended level (Mahdavi-Roshan et al., 2015), and that magnesium deficiency is associated with changes in the structure of apatite crystals in bones, a decrease in PTH, and thus a decrease in vitamin D levels (Mutlu et al., 2007).

In the present study, a reduction in the Mg content of the femurs was also observed in rats consuming a calcium-deficient diet (DEF group). This effect is probably attributable to the fact that Mg competes with calcium; it blocks the calcium channel, which reduces the intracellular calcium content (Houston, 2011). Mg competes with Ca in the formation of hydroxyapatite, forming an insoluble salt by binding to pyrophosphate (Navarro-González et al., 2009), and a high Mg content inhibits osteoblast differentiation, leading to a reduction in mineralization activity (Leidi et al., 2011). Matsuzaki et al. observed a similar relationship and reported that Ca supplementation reduces the Mg content in bones (Matsuzaki et al., 2005). However, some authors did not find this negative correlation between the Ca content in the diet and the Mg content in bones (Hernández-Becerra et al., 2017; 2020; Toba et al., 2000).

Although calcium lactate increased the Mg content in the liver, an innovative food product such as pumpkin enriched with calcium lactate contributed to an even higher accumulation of Mg in the liver. Enriched pumpkin also increased the Mg content in muscles compared with the OVX control group. The accumulation of Mg in the liver and muscles should therefore be attributable to the action of another component of the enriched pumpkin. During osmotic dehydration, inulin was used as an osmotically active substance, which is one of the factors increasing Mg absorption (Coudray et al., 2003; Schuchardt and Hahn, 2017). Inulin and other indigestible oligosaccharides affect both Ca and Mg metabolism by increasing the active and passive transport of these minerals (Scholz-Ahrens and Schrezenmeir, 2002), which may increase the Mg content in tissues.

Alendronate, a drug belonging to the group of bisphosphonates, is an oral therapeutic agent prescribed to women with postmenopausal osteoporosis for the inhibition of bone resorption and increase in bone formation, thereby protecting against bone mineral loss (Wang et al., 2017). Although alendronate does not affect the Mg content in body fluids (Buduneli et al., 2008; Shapses et al., 2011), bisphosphonates can affect Mg metabolism, for example, by impairing renal function, thereby increasing Mg excretion (Gröber, 2019). In general, the use of alendronate is safe and does not interfere with normal kidney function (Jamal et al., 2007; Sadowski et al., 2011); however, its long-term use may contribute to nephrotoxicity as one of the side effects (Benghuzzi et al., 2012; Miura et al., 2009). In the present study, a significant increase in the Mg content was observed in the kidneys after alendronate administration. Through the accumulation of calcium in the kidneys due to alendronate administration (described in the previous study (Wawrzyniak et al., 2022), adequate removal of Mg from the organism is disturbed. The changes in the kidneys can only be speculated, and to determine the changes caused by alendronate, the parameters of kidney functioning need to be analyzed. In this study, a synergistic effect of the ingredients of pumpkin and alendronate was observed on the increase in the Mg content in the femur.

The results of the study may contribute to the next ones, which could consist in enriching another raw material with calcium, e.g., apple or beetroot. Unfortunately, it is not advisable to use calcium-enriched pumpkin in clinical trials due to the very high accumulation of calcium in the kidneys, which was described in a previous article (Wawrzyniak et al., 2022).

STRONG POINTS AND LIMITATIONS

The strength of this study is its use of innovative products with potentially good calcium bioavailability in the protection and treatment of postmenopausal osteoporosis. We used a group with alendronate to compare the activity of the product with an antiosteoporotic drug. The dose of alendronate was adjusted weekly according to the body weight of the animals.

This study also has some limitations, which may have affected the results and limited the discussion. The volume of serum was not sufficient for the

determination of Mg. In addition, rats' urine was not collected, and thus Mg excretion was analyzed. The sham-operated group was not taken into account, whereas the results were compared with those of the nonoperated control group and the ovariectomized group with the standard diet.

CONCLUSIONS

Ovariectomy and intervention diets revealed various new observations regarding the effect of innovative calcium-rich foods on the Mg content. These results showed that (i) ovariectomy decreases the status of Mg content; (ii) deficiency of Ca in the diet and Ca-enriched pumpkin with alendronate improve the Mg content in bones; and (iii) alendronate promotes the accumulation of Mg in the kidneys. In postmenopausal women, both those treated and untreated with drugs and diet, magnesium status should be monitored.

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Research Article

Natalia Wawrzyniak, Anna Gramza-Michałowska, Joanna Suliburska*

Effect of pumpkin enriched with calcium lactate on iron status in an animal model of postmenopausal osteoporosis

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Abstract: The homeostasis of calcium (Ca) and iron (Fe) is disturbed during menopause. The present study aimed to determine the effects of Ca-enriched pumpkin on Fe status in ovariectomized rats. A total of 48 female Wistar rats were divided into six groups. One group was fed a standard diet (C), while the other five groups were ovariectomized and fed a standard diet (OVX), a calcium lactate diet (CaL_OVX), calcium lactate-enriched pumpkin (PCaL_OVX), calcium lactate and alendronate (CaL_OVX_B), and calcium lactate-enriched pumpkin and alendronate (PCaL_OVX_B), respectively. The nutritional intervention lasted 12 weeks and rats were euthanized. Tissue samples were collected, and the iron content in the samples was assessed. A comparison of all groups showed a reduction in iron concentrations in femurs, liver, hair, spleen, and kidneys in the ovariectomized groups than in the control group. The PCaL_OVX_B group had a significantly higher blood hemoglobin concentration than the control group. Moreover, spleen and liver Fe concentrations were the highest in PCaL_OVX and PCaL_OVX_B rats among the treated groups and were comparable with the control group. These results indicate that ovariectomy decreases Fe status in rats. Calcium lactate-enriched pumpkin with and without alendronate can increase Fe concentration in liver and spleen in ovariectomized rats.

Keywords: iron metabolism, ovariectomy, enriched pumpkin, calcium

1 Introduction

Menopause is associated with a reduction in estrogen levels, which leads to a significant decrease in bone mineral density, due to increased bone resorption [1]. Inhibition of endogenous ovarian function also leads to changes in the metabolism of minerals including iron (Fe). Studies that compared the serum of premenopausal and postmenopausal women showed significantly increased Fe concentrations and an increase in Fe stores at the end of menstruation [2,3], as indicated by up to threefold higher ferritin concentration [4]. In men, the accumulation of Fe in the body occurs from the stage of puberty. In the case of women, Fe accumulation takes place in the reproductive period, during which there is a systematic loss of Fe along with the monthly blood, and hence its accumulation begins only after menopause [5]. Fe status is determined from the serum concentration of ferritin, as well as hemoglobin (HGB), which also increases after menopause [6]. Changes in Fe metabolism affect the skeletal system; Fe overload results in the inhibition of bone formation and increased resorption, which is independent of the effect of estrogen [7]. Evidence shows that increased bone resorption activity is associated with an increase in urinary deoxypyridinoline in a rat model of postmenopausal osteoporosis and changes in bone architecture such as a decrease in the number of bone trabeculae and their thinning [8]. In contrast, reducing Fe overload may contribute to the normalization of bone resorption and formation [9], which can be achieved by using agents that control Fe stores, such as hepcidin [10]. If the concentration of Fe is so high that transferrin is unable to bind it, free Fe is deposited in the

* **Corresponding author: Joanna Suliburska,** Department of Human Nutrition and Dietetics, Faculty of Food and Nutrition Science, University of Life Sciences, 60-624 Poznan, Poland, e-mail: joanna.suliburska@up.poznan.pl
Natalia Wawrzyniak: Department of Human Nutrition and Dietetics, Faculty of Food and Nutrition Science, University of Life Sciences, 60-624 Poznan, Poland, e-mail: natalia.wawrzyniak@up.poznan.pl
Anna Gramza-Michałowska: Department of Gastronomy Sciences and Functional Foods, Faculty of Food and Nutrition Science, University of Life Sciences, 60-624 Poznan, Poland, e-mail: anna.gramza@up.poznan.pl
ORCID: Natalia Wawrzyniak 0000-0001-7074-9656;
Anna Gramza-Michałowska 0000-0002-0744-9033;
Joanna Suliburska 0000-0002-0937-8427

organs affecting them permanently [11], through various mechanisms, including increased oxidative stress [7].

During menopause, the calcium status is low in women [12]. In menopausal women, insufficient calcium intake results in lower Ca concentration [13] and higher serum parathyroid hormone levels, which increase bone turnover and accelerate resorption [12]. Therefore, a daily calcium dose of at least 1,200 mg is recommended for this group of women [14]. However, most of these women take only half of the recommended daily dose [15]. Thus, to effectively treat osteoporosis, an adequate amount of calcium should be included in the diet and ingredients increasing calcium bioavailability should also be taken [16]. It has been shown that dairy products, legumes, and fortified foods are good sources of calcium and help to maintain its balance [17].

Ca may inhibit the absorption of Fe by affecting the divalent metal transporter (DMT1) or inhibit the transfer of Fe ions to the blood. However, according to studies, this is only a short-term effect, and compensatory actions such as long-term Ca supplementation can prevent the negative effects associated with Fe metabolism [18]. Because both Fe and Ca play a role in bone metabolism and interact at the absorption level, it may be interesting to understand the effects of Ca-enriched food products on Fe status in rats. Pumpkin enriched with calcium is an innovative product, which is also enriched with inulin. Inulin is necessary in the process of osmotic dehydration as an osmotically active substance. Inulin is a fructan polysaccharide that has beneficial effects on the body, changing the composition of the microbiota, stimulating immune functions, reducing constipation. Inulin also affects the bioavailability of minerals [16]. It forms complexes with iron in the intestinal tract, which leads to an increase in its absorption by extending the time of residence in the intestine. It also increases the bioavailability of calcium by stimulating the growth of colon cells, thereby increasing the absorption surface and stimulating the expression of calcium-binding proteins [16,17].

Therefore, this study aimed to determine the effects of Ca-enriched pumpkin on Fe status in ovariectomized rats.

2 Methods

2.1 Materials and reagents

Calcium lactate and inulin were purchased from Agnex (Białystok, Poland). Pumpkin (yellow melon, *Cucurbita maxima*) was obtained from an organic farm (with the consent of the land owner). Dietary ingredients (minerals, vitamins, and macroingredients) were procured from Sigma-Aldrich (Darmstadt, Germany), Hortimex (Konin, Poland), and Warchem (Warszawa, Poland).

2.2 Osmotic dehydration

Through osmotic dehydration, pumpkin tissue was enriched with calcium lactate. Briefly, calcium lactate was dissolved in an inulin solution (5:1 with distilled water) to make up 5% of the content. Frozen pumpkin flesh cubes were added to this mixture in a 1:5 ratio. The jars containing pumpkin cubes were shaken in a 50°C water bath for 2 h. Then, enriched pumpkin was drained and freeze-dried. The prepared pumpkin powder was added to the diets of rats [19].

2.3 Animals

A total of 48 female Wistar rats aged 12 weeks were obtained from the Center for Advanced Technologies in Greater Poland, University of Adam Mickiewicz in Poznań, Poland. All animal experiments were carried out following the guidelines for the use and care of laboratory animals according to the EU Directive 2010/63/EU. The study was approved by the Local Ethics Committee in Poznań (no. 34/2019).

2.4 Experimental protocols

During the experiment, the rats were fed the standard AIN-93M diet [20]. The animals were divided into six groups of eight each. The average initial body weight of rats was 267.7 ± 16.1 g. Five of these groups (40 rats) were ovariectomized, and a 12-week nutritional intervention was introduced after the recovery period (2 weeks after ovariectomy). One of the ovariectomized (OVX) groups and the control group (C) received the standard AIN-93M diet without modifications, whereas the other four groups received one of the following modified diets: calcium lactate diet (CaL_OVX), calcium lactate-enriched pumpkin (PCaL_OVX), alendronate and calcium lactate (CaL_OVX_B), or calcium lactate-enriched pumpkin and alendronate (PCaL_OVX_B). The dietary components are summarized in the previous study [19]. The design of the experiment is presented in Figure 1. Dietary supplements were added such that the calcium content remained the

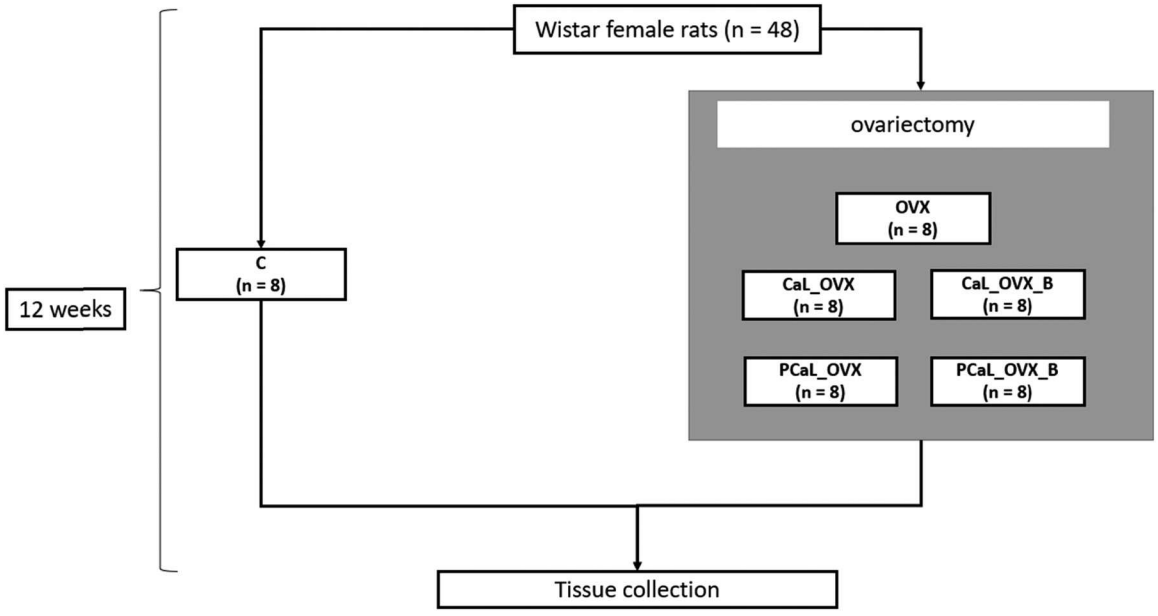

Figure 1: Scheme of the study. C, control group; OVX, ovariectomized group; CaL_OVX, ovariectomized group receiving calcium lactate; CaL_OVX_B, ovariectomized group receiving calcium lactate and alendronate; PCaL_OVX, ovariectomized group receiving calcium lactate-enriched pumpkin; PCaL_OVX_B, ovariectomized group receiving calcium lactate-enriched pumpkin and alendronate.

same (0.5% Ca in each of modified diet), while the dose of alendronate was adjusted weekly based on the actual body weight of the animals (3 mg/kg of body mass). The rats were provided food and water ad libitum, and the consumption was recorded daily. Body weight was measured weekly and at the end of the experiment using a Bruker LF90II body composition analyzer. After 12 weeks, the rats were euthanized by decapitation. Whole blood was collected for the determination of HGB. Femurs, spleen, pancreas, heart, liver, brain, kidneys, and muscles were isolated for analysis. Hair was collected from the interscapular area of all rats. Tissues were weighed, washed with saline, and frozen at −80°C.

2.5 Fe analysis in diets

To determine the Fe content in the studied diets, 1 g of each diet was burned at 450°C in a muffle furnace until mineralization. Then, the samples were dissolved in 1 mol/l nitric acid (Merck, Kenilworth, NJ, USA). The dissolved samples were diluted with appropriate amounts of deionized water (AAS-3, Carl Zeiss, Jena, Germany), and their mineral content was determined using flame atomic absorption spectrometry (AAS). The method was validated using a certified reference material (BCR191, Sigma-Aldrich, St. Louis, MO, USA) with an accuracy of 92%. All samples were analyzed in triplicate.

2.6 HGB determination in blood

Whole-blood HGB determination was carried out by a commercial laboratory (Alab, Poznań, Poland).

2.7 Fe analysis in tissues

Samples containing pure nitric acid (Merck, Kenilworth, NJ, USA) were mineralized in a Microwave Digestion System (Speedwave Xpert, Berghof, Eningen, Germany) to determine the Fe content in tissues. After digestion, the samples were diluted with deionized water. Flame AAS was used to determine the content of minerals (AAS-3; Carl Zeiss, Jena, Germany) at a wavelength of $\lambda = 248.3$ nm. The method was validated using bovine liver as certified reference material (1577C; Sigma-Aldrich, St. Louis, MO, USA) with an accuracy of 91%.

2.8 Statistical analysis

Statistical analyses were performed using the Statistica program (StatSoft, Tulsa, OK, USA). The Shapiro–Wilk test was used to determine the normality of the distribution of the variables. One-way analysis of variance with Tukey's post hoc test was used to identify the statistical

Table 1: Daily intake, iron intake, and body mass gain (mean and standard deviation)

Parameter	Group					
	C	OVX	CaL_OVX	CaL_OVX_B	PCaL_OVX	PCaL_OVX_B
Daily intake of diet (g)	25.08 ± 0.63	25.11 ± 1.70	25.90 ± 0.55	25.66 ± 2.29	24.31 ± 1.26	24.84 ± 2.29
Fe diet content (μg/g dm)	35.77 ± 0.59	35.77 ± 0.59	35.77 ± 0.33	35.77 ± 0.33	35.67 ± 0.91	35.67 ± 0.91
Fe daily intake (μg)	901.01 ± 23.88	896.77 ± 65.41	938.23 ± 25.99	903.02 ± 66.53	869.48 ± 41.31	869.11 ± 97.35
Body mass gain (g)	55.45 ± 20.33^{a}	114.71 ± 32.59^{c}	105.75 ± 30.15^{bc}	106.00 ± 27.82^{bc}	71.63 ± 10.39^{ab}	74.38 ± 20.84^{ab}

C, control group; OVX, ovariectomized group; CaL_OVX, ovariectomized group receiving calcium lactate; CaL_OVX_B, ovariectomized group receiving calcium lactate and alendronate; PCaL_OVX, ovariectomized group receiving calcium lactate-enriched pumpkin; PCaL_OVX_B, ovariectomized group receiving calcium lactate-enriched pumpkin and alendronate; dm, dry mass.
^{a,b,c}Significant differences between groups ($p < 0.05$).

differences between the groups, and Student's t-test was used to compare two groups ($p < 0.05$).

3 Results

The results of the analysis are presented in Tables 1–3. It was observed that Fe content in diets did not differ between the studied groups (Table 1). Similarly, there were no differences in daily Fe consumption between the groups. However, some differences were observed in weight gain. Ovariectomy caused a significant increase in the weight gain of rats, which was minimized by pumpkin enriched with calcium lactate with or without alendronate (PCaL_OVX and PCaL_OVX_B groups).

Table 2 presents the effect of ovariectomy and interventional diets on tissue iron content and whole-blood HGB concentration.

It was noted that ovariectomy did not influence the concentration of HGB in whole blood, while enriched pumpkin and alendronate diets (PCaL_OVX_B group) caused a significant increase in HGB concentrations compared to the control group. Ovariectomy significantly reduced the iron content in bone, hair, and kidneys compared to the control group, whereas modified diets did not result in any change. Although ovariectomy had no effect on pancreatic iron content, the administration of calcium lactate reduced pancreatic iron levels compared to the control group. In contrast, ovariectomy significantly reduced spleen and liver iron levels in rats, while enriched pumpkin reversed this effect, which was enhanced by the addition of alendronate (PCaL_OVX and PCaL_OVX_B

Table 2: HGB concentration in whole blood and iron content in tissues (mean and standard deviation)

Tissue	Group					
	C	OVX	CaL_OVX	CaL_OVX_B	PCaL_OVX	PCaL_OVX_B
HGB (g/dl)	15.29 ± 0.67^{a}	15.65 ± 0.65^{ab}	16.19 ± 0.74^{ab}	16.00 ± 0.57^{ab}	16.00 ± 0.79^{ab}	16.31 ± 0.56^{b}
Femur (μg/g dm)	87.52 ± 9.43^{b}	55.86 ± 10.04^{a}	69.95 ± 9.19^{a}	59.80 ± 10.14^{a}	66.20 ± 7.72^{a}	58.24 ± 16.91^{a}
Pancreas (μg/g dm)	111.49 ± 11.47^{b}	99.93 ± 11.89^{ab}	89.85 ± 12.56^{a}	94.74 ± 10.63^{ab}	105.41 ± 13.48^{ab}	102.66 ± 13.57^{ab}
Hair (μg/g dm)	176.09 ± 16.26^{b}	125.24 ± 8.73^{a}	153.95 ± 25.75^{ab}	145.77 ± 24.33^{a}	133.64 ± 19.57^{a}	140.84 ± 16.69^{a}
Spleen (mg/g dm)	10.30 ± 2.07^{d}	5.15 ± 0.89^{a}	6.54 ± 1.68^{ab}	7.29 ± 1.13^{abc}	7.78 ± 1.25^{bc}	8.93 ± 1.44^{cd}
Liver (mg/g dm)	1.61 ± 0.29^{bc}	1.10 ± 0.18^{a}	1.39 ± 0.14^{ab}	1.20 ± 0.11^{a}	1.66 ± 0.25^{bc}	1.84 ± 0.20^{c}
Heart (μg/g dm)	515.93 ± 34.91	535.40 ± 37.33	536.48 ± 34.73	558.14 ± 28.47	524.03 ± 39.35	541.47 ± 32.44
Brain (μg/g dm)	130.72 ± 16.42	145.25 ± 20.65	134.68 ± 13.12	136.30 ± 20.57	135.06 ± 14.96	138.94 ± 13.54
Muscle (μg/g dm)	91.27 ± 15.53^{b}	79.71 ± 14.65^{b}	78.15 ± 11.47^{ab}	81.43 ± 12.31^{b}	59.14 ± 6.44^{a}	81.64 ± 16.00^{b}
Kidney (μg/g dm)	760.39 ± 124.34^{b}	578.32 ± 57.19^{a}	690.52 ± 62.72^{ab}	663.06 ± 43.93^{ab}	708.21 ± 104.78^{ab}	664.24 ± 115.31^{ab}

C, control group; OVX, ovariectomized group; CaL_OVX, ovariectomized group receiving calcium lactate; CaL_OVX_B, ovariectomized group receiving calcium lactate and alendronate; PCaL_OVX, ovariectomized group receiving calcium lactate-enriched pumpkin; PCaL_OVX_B, ovariectomized group receiving calcium lactate-enriched pumpkin and alendronate; HGB, hemoglobin; dm, dry mass.
^{a,b,c,d}Significant differences between groups ($p < 0.05$).

Table 3: Significant effect of ovariectomy, Ca-enriched pumpkin, and alendronate on iron content in tissues

Tissue	Ovariectomy	Enriched pumpkin	Bisphosphonate	Bisphosphonate + enriched pumpkin
Femur	↓			
Pancreas		↑		
Hair	↓			
Spleen	↓			↑
Liver	↓	↑	↓	↑
Heart				
Brain				
Muscle		↓		
Kidney	↓			

Compared groups: ovariectomy, C:OVX; enriched pumpkin, CaL_OVX:PCaL_OVX; bisphosphonate, CaL_OVX:CaL_OVX_B; bisphosphonate + enriched pumpkin, CaL_OVX:PCaL_OVX_B. C, control group; OVX, ovariectomized group; CaL_OVX, ovariectomized group receiving calcium lactate; CaL_OVX_B, ovariectomized group receiving calcium lactate and alendronate; PCaL_OVX, ovariectomized group receiving calcium lactate-enriched pumpkin; PCaL_OVX_B, ovariectomized group receiving calcium lactate-enriched pumpkin and alendronate.

groups). However, ovariectomy did not affect the iron content in the muscles, whereas the muscle's Fe concentration was decreased in rats fed enriched pumpkin compared to groups C and OVX.

The study also analyzed the effect of a simple factor on Fe content in tissues. Table 3 shows significant changes in Fe content in tissues caused by ovariectomy and feeding with calcium lactate-enriched pumpkin and alendronate. The effect of ovariectomy was investigated by comparing the control group and the ovariectomized group (C:OVX). The effect of Ca-enriched pumpkin on Fe content was investigated by comparing the group receiving calcium lactate and the group receiving calcium lactate-enriched pumpkin (CaL_OVX:PCaL_OVX). The effect of alendronate was determined by comparing the calcium lactate group and the calcium lactate + alendronate group (CaL_OVX:-CaL_OVX_B), while the combined effect of enriched pumpkin with alendronate was analyzed by comparing the calcium lactate group and the enriched pumpkin + alendronate group (CaL_OVX:PCaL_OVX_B).

We observed that ovariectomized groups had reduced Fe content in bones, hair, spleen, liver, and kidneys compared to the control group. The enriched pumpkin group (PCaL_OVX) showed higher Fe content in the pancreas and liver, but lower Fe content in muscles compared to the calcium lactate group (CaL_OVX). The group receiving bisphosphonates + calcium lactate (CaL_OVX_B) had reduced Fe content in the liver in comparison to the CaL_OVX group. The group fed bisphosphonates + enriched pumpkin (PCaL_OVX_B) showed higher spleen and liver Fe content compared to the CaL_OVX group.

4 Discussion

This study confirmed that ovariectomy losses in the majority of the organs in rats. In contrast, pumpkin enriched with calcium lactate with or without alendronate prevented the loss of Fe in the liver and spleen in ovariectomized rats. Moreover, we found that feeding with Ca-enriched pumpkin with or without alendronate more efficiently reduced Fe losses in organs compared to calcium lactate used alone.

Menopause causes an increase in iron content in women; however, our study showed the opposite effect, as indicated by a decrease in Fe content after ovariectomy. Liu et al. observed a lack of Fe overload in ovariectomized rats in their study, in which ovariectomy did not cause any changes in serum iron concentration in rats [21]. It can be assumed that the decrease in Fe concentrations in the body is related to increased body weight after ovariectomy and thus increased blood volume and increased Fe losses associated with excessive body weight [22]. It is important to regulate Fe status in people with obesity, mainly due to increased concentration of leptin, which is biologically similar to interleukin 6, and whose increase causes an increase in the secretion of hepcidin from the liver [23]. The excessive adiposity is associated with disturbances of iron homeostasis due to elevated hepcidin expression and increased ferritin level. This dysregulation of iron metabolism in obesity increases the risk of the iron overload [24–26]. It seems that the consumption of enriched pumpkin may be beneficial in people with obesity because: first, it is a source of well-absorbed calcium and, second, it may reduce the risk of iron overload.

Hepcidin, on the other hand, regulates iron status in the body, as it interacts with ferroportin, a protein that controls Fe efflux from cells [27]. High levels of hepcidin block intestinal iron absorption, resulting in erythropoiesis and anemia due to reduced iron content [28]. A decrease in iron content in tissues observed in this study can also be caused by impaired Fe absorption in the duodenum and impaired Fe transport to the blood [29].

In the present study, we observed that calcium-enriched pumpkin increased Fe content in tissues in ovariectomized rats. Pumpkin was enriched not only with calcium but also with inulin – an osmotically active substance necessary for osmotic dehydration. Inulin is a polysaccharide and a prebiotic with beneficial effects on the human body [30]. A study showed that inulin significantly increased Fe absorption in children and adolescents by reducing Fe intake via the regulation of intestinal microbiome and increasing the count of *Bifidobacterium* [31] and *Lactobacillus* and decreasing *Clostridium* bacteria [32]. Furthermore, inulin increases the absorption surface and stimulates the formation of short-chain fatty acids (SCFAs) in the intestines [33], which contribute to strengthening the absorbent surface by the proliferation of epithelial cells [34]. SCFAs also reduce pH, promoting an acidic environment that favors the conversion of iron to its absorbable ferrous state [35]. Studies have shown changes in the composition of intestinal microbiota in the presence of inulin, but there is no evidence of an increase in Fe absorption in women with low iron status [36]. Recently, it was reported that by modulating the intestinal microbiota, inulin influences the metabolism of organs involved in maintaining energy homeostasis, including skeletal muscles [37]. Increased production of SCFAs, which results from the action of inulin, leads to the activation of the AMP protein in the muscles [38]. In contrast, muscle protein kinase activated by AMP takes part in the inhibition of Fe-dependent cell apoptosis (ferroptosis) caused by energy stress [39]. This relationship between AMP and iron may partly explain the lowest Fe concentration observed in muscles in the PCaL_OVX group in this study.

Our study showed that pumpkin enriched with calcium and inulin increases Fe content in soft tissues, while it has no effect on Fe saturation in bones. This finding is in agreement with the study by Jolliff and Mahan who observed a significant increase in Fe content in the liver after the addition of inulin to the diet [40]. It has been found that inulin increased the formation of the Fe transporter DMT1, ferroportin, and ferritin, by increasing the rate of Fe absorption in the duodenum and liver of chickens [41]. Furthermore, inulin reduced the expression of pro-inflammatory genes and increased the expression of genes encoding Fe storage proteins (CYBRD1, FTL, HEPH, HIF1, LTF, UBE2D1) in the livers of young pigs, with the simultaneous lack of inulin's effect on the expression of Fe transporters in the intestine (DMT-1 and ferroportin) and the increased expression of Fe regulators, supporting a feedback loop that would prevent Fe overloading [42].

Consumption of inulin positively influences Fe metabolism, improving Fe bioavailability, and also increases the concentration of HGB in the blood, as has been observed in women of reproductive age [43] and in animals [44,45].

In this study, increased iron concentration in the liver was associated with lower weight gain in groups fed with enriched pumpkin. It seems that enriched pumpkin reduced fat content, and this effect might be accompanied by lower inflammation and hepcidin level, resulting in improved iron status [46]. After ovariectomy, iron transfer to bones may have been inhibited by interaction with calcium.

Lowering bone iron content may have clinical implications. Iron is involved in two critical processes related to bone health. The first of them is the production of collagen through the hydroxylation of procollagen, the second – is the metabolism of vitamin D, by regulating the synthesis of this vitamin with cytochrome P450 having heme in its molecule [47]. Iron deficiency, therefore, leads to a decrease in bone mineral density, increasing the rate of bone turnover [48]. In contrast, iron overload in postmenopausal women leads to reduced bone mineral density, which correlates with blood ferritin levels [49]. Excessive deposition of iron in the bones also leads to increased bone turnover [50].

We observed that alendronate decreased Fe level in the liver compared to Ca lactate used alone. However, Ca-enriched pumpkin offset the adverse effect of alendronate in rats. Human and *in vitro* studies have not confirmed the effect of alendronate on Fe content in the liver or on blood morphology [51,52]. The possible explanation for the differences in Fe concentrations is the level of alendronate in the blood [53,54]. Jing et al. showed that a combination of alendronate and lactic acid derivatives may increase the drug's bioavailability and thus its effect on mineral metabolism [55]. However, another study showed that when consumed with a meal (mainly fruit) the bioavailability of alendronate decreased drastically. Unfortunately, in our study, we did not analyze alendronate concentration in the blood of rats. However, it has been found that bisphosphonates are effective in the treatment of thalassemia-associated osteoporosis [56]. Moreover, some bisphosphonates, such as risedronate, have been shown to decrease the serum ferritin level in postmenopausal women with osteoporosis [57].

The present study showed that calcium-enriched pumpkin increased Fe content in liver and spleen and HGB levels in ovariectomized rats. However, these findings may point to possible side effects of enriched pumpkin in postmenopausal women. After menopause, ferritin and iron levels usually increase in women, so calcium-enriched pumpkin may cause iron accumulation and accelerate bone loss in the body. Further studies are needed to analyze the positive and side effects of this novelty product in postmenopausal women. Nevertheless, studies should explore the potential application of calcium-enriched pumpkin in iron deficiency conditions.

4.1 Strong points and limitations

The strength of the research is the use of a new product, namely pumpkin enriched with calcium lactate. We evaluated for the first time the effect of enriched pumpkin on iron status in ovariectomized rats. In addition, we analyzed the effect of calcium lactate without plant matrix and the effect of alendronate on many parameters of iron metabolism.

This study also has a few limitations, which may have affected the results. The volume of serum collected from rats was not sufficient for the determination of Fe or alendronate. Moreover, only selected parameters of iron metabolism were analyzed; for instance, we did not analyze the levels of ferritin and hepcidin in the blood. In addition, rats' urine was not collected, so we could not analyze the Fe content in feces and thus calculate the amount of excreted Fe. The sham-operated group was not taken into account, and the results were compared with those of the ovariectomized group fed with the standard diet and the nonoperated control group.

5 Conclusion

Based on the obtained results, we can conclude that: (i) ovariectomy decreases the Fe status in rats and (ii) calcium lactate-enriched pumpkin with or without alendronate increases the Fe content in liver and spleen in ovariectomized rats.

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Ethics approval: All experimental procedures were performed in accordance with the EU Directive 2010/63/EU for animal experiments. Approval for the study was obtained from the Local Ethics Committee in Poznań (no. 34/2019). The reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Oświadczenia współautorów prac tworzących cykl

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prof. dr hab. n. med. i n. o zdr. Joanna Sulibursk
Katedra Żywienia Człowieka i Dietetyki
Uniwersytet Przyrodniczy w Poznaniu.

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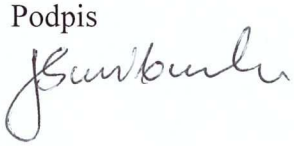

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Uniwersytet Przyrodniczy w Poznaniu.

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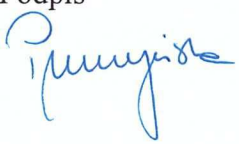

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Katedra Fizjologii, Biochemii i Biostruktury Zwierząt
Uniwersytet Przyrodniczy w Poznaniu

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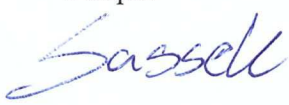

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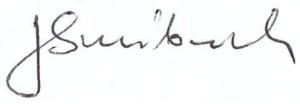

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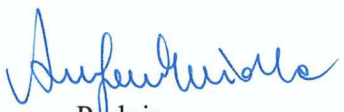

Podpis

Poznań, 23.05.2023

dr n med. Paweł Kurzawa
Zakład Patologii Onkologicznej
Uniwersytet Medyczny w Poznaniu

OŚWIADCZENIE

Oświadczam, że w pracy Wawrzyniak N., Gramza-Michałowska A., Kurzawa P., Kołodziejski P., Suliburska J. Calcium carbonate□enriched pumpkin affects calcium status in ovariectomized rats. Journal of Food Science and Technology. 2023;60(4):1402-1413 mój udział polegał na pomocy w ocenie histopatologicznej oraz pomocy w dalszej analizie.

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Podpis

Paweł Kurzawa.

Poznań, 11.05.2023

dr hab. Paweł Kołodziejski
Katedra Fizjologii, Biochemii i Biostruktury Zwierząt
Uniwersytet Przyrodniczy w Poznaniu

OŚWIADCZENIE

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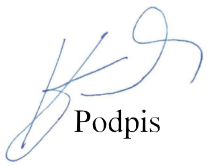

Podpis

Poznań, 11.05.2023

prof. dr hab. n. med. i n. o zdr. Joanna Suliburska
Katedra Żywienia Człowieka i Dietetyki
Uniwersytet Przyrodniczy w Poznaniu.

OŚWIADCZENIE

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Podpis

Poznań, 11.05.2023

prof. dr hab. Anna Gramza-Michałowska
Katedra Technologii Gastronomicznej i Żywności Funkcjonalnej
Uniwersytet Przyrodniczy w Poznaniu.

OŚWIADCZENIE

Oświadczam, że w pracy Wawrzyniak N., Gramza-Michałowska A., Sulibурska J. Effect of ovariectomy and calcium enriched pumpkin on magnesium status in rats. Acta Scientiarum Polonorum Technologia Alimentaria. 2022;21(4):439-448 mój udział polegał na opracowaniu innowacyjnego produktu (dynia wzbogacona w wapń), pozyskaniu źródeł finansowych na przeprowadzenie badań, akceptacji ostatecznej wersji manuskryptu.

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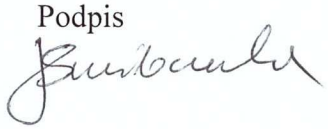
Podpis



Poznań, 11.05.2023

prof. dr hab. Anna Gramza-Michałowska
Katedra Technologii Gastronomicznej i Żywności Funkcjonalnej
Uniwersytet Przyrodniczy w Poznaniu

OŚWIADCZENIE

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Podpis

Poznań, 11.05.2023

prof. dr hab. n. med. i n. o zdr. Joanna Suliburska
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Uniwersytet Przyrodniczy w Poznaniu.

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Podpis

Zgoda na badania na zwierzętach

UCHWAŁA NR **34/2019**

z dnia 6.09.2019r.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Poznaniu

§ 1

Na podstawie art. 48 ust. 1 pkt. 1 ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: **„Ocena możliwości zastosowania wzbogaconego miąższu dyni (*Cucurbita L.*) w prewencji i wspomaganiu leczenia osteoporozy pomenopauzalnej"** z dnia **26.08.2019r.**, złożonego przez **Uniwersytet Przyrodniczy w Poznaniu, Wydział Nauk o Żywności i Żywieniu**, adres **ul. Wojska Polskiego 31; 60-624 Poznań** zaplanowanego przez **dr hab. Joannę Sulibursk**ą.

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w §1 , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: **[PB6] badania podstawowe; układ mięśniowo-szkieletowy**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **umiarkowany**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków: **Szczur wędrowny *(Rattus norvegicus)*, stado niekrewniacze Wistar, wiek 12 tygodni 110 samic**
4. Doświadczenia będą przeprowadzane przez: **dr hab. Joannę Sulibursk**ą, **dra Pawła Antoniego Kołodziejskiego, mgr Małgorzatę Tubacką,**
5. Doświadczenie będzie przeprowadzane w terminie **od 06.01.2020r. do 20.12.2021r.**
6. Doświadczenie będzie przeprowadzone w ośrodku: **nie dotyczy**
7. Doświadczenie będzie przeprowadzone poza ośrodkiem **nie dotyczy**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ....., w sposób: **nie dotyczy**
9. Doświadczenie **nie zostanie** poddane ocenie retrospektywnej w terminie do ... miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.

§ 3

Uzasadnienie:

Celem naukowym doświadczenia jest określenie wpływu dyni (*Cucurbita L.)* wzbogaconej w wapń organiczny i nieorganiczny na gospodarkę wapnia i metabolizm kostny u szczurów po owariektomii. Osteoporoza pomenopauzalna spowodowana jest ustaniem endokrynnych funkcji gruczołów jajnikowych, co prowadzi m.in. do utraty masy kostnej, upośledzenia wchłaniania wapnia w przewodzie pokarmowym oraz obniża resorpcję zwrotną wapnia w nerkach. Choroba ta występuje u ponad 30% kobiet po 50 roku życia. Uważa się, że odpowiednio zbilansowana dieta może stanowić istotny element w profilaktyce i hamowaniu jej rozwoju. Spożycie wapnia jest kluczowe w profilaktyce i hamowaniu rozwoju osteoporozy, ponieważ jest on składnikiem budującym szkielet człowieka, jednocześnie potwierdza się korzystne działanie związków o właściwościach antyoksydacyjnych, szczególnie karotenoidów i flawonoidów. Cel badań został jasno sformułowany i uzasadniony, a wniosek nie wzbudza zastrzeżeń natury etycznej. Wnioskodawca zapewni właściwe warunki utrzymania zwierząt. Procedury oraz zasady 3R zostały opisane prawidłowo.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
Uniwersytet Przyrodniczy w Poznaniu
60-637 Poznań, ul. Wołyńska 35
tel. 61-846 60 83, tel. 798 641 366

(Pieczęć lokalnej komisji etycznej)

Podpis przewodniczącego komisji

.....

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej do Spraw
Doświadczeń na Zwierzętach
w Poznaniu
dr hab. Katarzyna Szkudelska

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:

1) Użytkownik,
2) a/a

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UCHWAŁA NR **58/2019**

z dnia 03.01.2020r.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Poznaniu

§ 1

Lokalna komisja etyczna po rozpatrzeniu wniosku pt.: ***„Ocena możliwości zastosowania wzbogaconego miąższu dyni (Cucurbita L.) w prewencji i wspomaganiu leczenia osteoporozy pomenopauzalnej"*** z dnia **20.12.2019r.**, złożonego przez **Uniwersytet Przyrodniczy w Poznaniu, Wydział Nauk o Żywności i Żywieniu,** adres **ul. Wojska Polskiego 31, 60-637 Poznań,** zaplanowanego przez **prof. dr hab. Joannę Suliburską** a dotyczącego:

dodatkowych osób przeprowadzających doświadczenia

w ramach wydanej przez komisję zgody uchwałą **nr 34/2019 w dn. 06.09.2019r.**

WYRAŻA ZGODĘ

na dokonanie zmian w zakresie określonym poniżej.

§ 2

1. Najwyższy stopień dotkliwości proponowanych procedur po zatwierdzonych zmianach to: **nie dotyczy**
1. Zespół prowadzący doświadczenia rozszerza się o następujące osoby (nazwisko i imię, nazwa użytkownika):, **mgr Natalię Wawrzyniak i mgra Bartosza Kulczyńskiego Uniwersytet Przyrodniczy w Poznaniu, Wydział Nauk o Żywności i Żywieniu**
2. Doświadczenie będzie przeprowadzane w terminie od.... do.....**-nie dotyczy**

§ 3

Uzasadnienie:

Pani mgr Natalia Wawrzyniak i Pan mgr Bartosz Kulczyński ukończyli szkolenie dla osób uczestniczących organizowane przez Wydział Nauk o Żywności i Żywieniu UPP; 26.10-18.11.2019r. i posiadają stosowne wyznaczenia.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1

LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
Uniwersytet Przyrodniczy w Poznaniu
60-637 Poznań, ul. Wołyńska 35
tel. [illegible] 841 366

(Pieczęć lokalnej komisji etycznej)

Podpisy przewodniczącego komisji

PRZEWODNICZĄCY
Lokalnej Komisji Etycznej do Spraw
Doświadczeń na Zwierzętach
w Poznaniu
dr hab. Katarzyna Szkudelska

Otrzymuje Użytkownik

Pouczenie:

Od decyzji komisji można wnieść odwołanie do Krajowej Komisji Etycznej w terminie 14 od dnia otrzymania uchwały.

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu