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## Developing a commercially viable process for an Active Pharmaceutical Ingredient, Challenges, Myths and Reality in the Art of Process Chemistry

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# **Development of a Combined Bovine Colostrum and Immune-Stimulatory Carbohydrate Nutraceutical for Enhancement of Endogenous Stem Cell Activity**

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## **Abstract:**

There is increasing interest in the use of functional foods to activate natural stem cell associated repair pathways resulting in improved health and well-being. Bovine Alpha Lipid colostrum, a proprietary formulation, induced stem cell associated cytokines and growth factor, GCSF, IL-6 and VEGF and also enhanced the differentiation of bone marrow stem cells *in vitro*. To boost colostrum activity, commercially available natural extracts of immune stimulatory carbohydrates were screened for their capacity to induce GCSF and IL-6. Combining selected extracts with colostrum, showed synergistic cytokine induction *in vitro*. In a mouse feeding study, a trial formulation of Alpha Lipid colostrum combined with selected carbohydrate extracts, was shown to increase the frequency of bone marrow hematopoietic stem cells to a greater extent that was seen with the colostrum alone, as assessed by CFU assay. Furthermore, a pilot human study showed there were alterations in circulating hematopoietic and mesenchymal/stromal stem cell numbers following consumption of the trial formulation compared to subjects receiving a placebo supplement. These data indicate that is possible to influence the endogenous stem cell niche by oral supplementation with a combination of colostrum and highly potent sources of immune stimulatory carbohydrates.

**Key words:** bovine colostrum, feeding studies, functional foods, food supplement, gut-associated lymphoid tissue, immune stimulatory carbohydrates, hematopoietic stem cells, mesenchymal/stromal stem cells

## **Introduction:**

Maintaining good health involves ongoing tissue and organ repair processes which center on effective activation, mobilization and differentiation of progenitor cells that reside within specialized niches

throughout the body, in particular the bone marrow. There is increasing evidence to support the hypothesis that, certain types of functional foods can enhance the body's regenerative capacity through their ability to stimulate immune cell pathways that activate stem cell niches and also counteract oxidative stress and inflammation [1-5]. Such ability to enhance endogenous repair pathways would not only be of benefit to overall health status, but may also provide support to individuals receiving laboratory-derived stem cells as part of regenerative therapy.

Colostrum contains preformed growth factors and cytokines that may support activation of the bone marrow stem cell niche directly following absorption into the blood stream. It also contains other proteins that may stimulate the stem cell niche indirectly via activation of the innate immune system [6-11]. Colostrum produced by different manufacturing processes results in varying bioactivity, depending on processing methods employed after collection. It may be frozen after collection, ultra filtered, spray dried or freeze dried, all of which have different effects on the multiple active constituents. The colostrum used in this study was Alpha Lipid colostrum, a patented formulation that combines colostrum with a phospholipid delivery system that protects colostrum during processing as well as following consumption [12]. The object of these studies was to determine whether Alpha Lipid colostrum has the capacity to stimulate the production of hematopoietic/stem cell-related cytokines, IL-6 and GCSF and also the important growth factor VEGF. Further, we determined whether colostrum could alter cellular parameters associated with bone marrow derived stem cells, such as survival and differentiation using *in vitro* assay systems.

Notwithstanding the ability of bovine colostrum to enhance endogenous stem cell activity, there is further scope to enhance this type of activity by exploiting the activities of natural ingredients that are able to stimulate myeloid immune cells associated with the gut. One source of orally bioavailable myeloid cell-activating substances are immune stimulatory carbohydrates, some of which are a major component in Chinese medicine [13-17]. Immune stimulatory carbohydrates and their complex polymers, polysaccharides are a well-known class of compounds that readily target the carbohydrate recognition receptors on myeloid cells localized within the gut-associated lymphoid tissue (GALT), the largest immune network in the body [18, 19]. Accordingly, dietary carbohydrates from a wide range of sources have been shown to play multiple roles in potentiating immune responses after oral as well as parental delivery [15, 16, 20-22]. Different routes of uptake are involved with particulate, insoluble carbohydrate most likely directly taken up by GALT-associated cells, which then migrate, to secondary lymphoid organs, and water-soluble carbohydrates such as fungal-derived glucans being directly absorbed into the blood stream. Following carbohydrate binding, cell signaling is induced that results in local and systemic production of cytokine, chemokine and growth factors. As a result, gut-resident immune cells become activated and migrate to secondary lymphoid tissues as well as send signals to the bone marrow that activate stem cell release as part of homeostatic feedback mechanisms. These cells in turn can migrate to damaged tissues and support cellular repair mechanisms [23-25]. There are several different types of cellular carbohydrate receptors. A highly studied receptor is the  $\beta$ -glucan receptor, Dectin-1 [26] that binds  $\beta$  1-3, 1-6 glucans. Other cell-associated carbohydrate recognition

receptors are macrophage-inducible C-type lectin (Mincle), the dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), DC-SIGN related (DC-SIGNR) and the mannose binding lectin (MBL) [27-29]. All of these carbohydrate receptors are widely available and are predominantly expressed on the surface of myeloid cells of the monocyte/macrophage and neutrophil lineages.

*In vitro* bioassays were used to screen a range of different types and sources of naturally occurring carbohydrates for their relative capacity to induce stem cell-associated cytokines. Colostrum and a combination of colostrum and selected carbohydrates were tested in a mouse oral feeding study the ability to influence the bone marrow stem cell niche. This activity was measured by quantifying the frequency of hematopoietic progenitor cells, an indicator of bone marrow stem cell proliferation, using the CFU assay. Using this system we were able to detect an increased frequency of stem cells and this correlated with an overall increased proportion of bone marrow cells detected in the replicative phase of the cell cycle (S/G2). A pilot human study to determine the effects of this combination on circulating, peripheral blood stem cells demonstrated modulation of the number of circulating stromal and hematopoietic stem cell lineages. These findings demonstrate that nutraceutical supplementation can modulate stem cell activity and add support to the concept that certain functional foods contribute towards physical well-being and prevention of age-related diseases.

## **MATERIALS AND METHODS**

### **Animals**

Female CD-1 mice (8-12 weeks old) were used for generation of *in vitro* bone marrow cultures and 12-week mice were used for the oral feeding study. The animals were maintained according to the principles and guidelines of the ethical committee for animal care at Auckland University. The University of Auckland ethics approval number for this study is C877.

### **Alpha Lipid colostrum**

Colostrum used was Alpha Lipid colostrum powder, obtained from New Image Group, NZ. This is a patent protected formulation comprising bovine colostrum coated with the alpha lipids, sphingomyelin, ganglioside and phospholipid, that have been extracted from milk. This method of coating the colostrum with lipid mixture during spray drying results in increased dispersibility of the colostrum and also acts as an absorption enhancer, improving the penetration of bioactive components [12].

### **Immune-stimulatory carbohydrates**

Shiitake and Reishi Mushroom betaglucan extracts, Fructus jujube root extract and Scuterllariac were obtained from Suzhou Vitajoy Biotech, China. GanoPolyC, an extract containing *Ganoderma lucidum* and *Cordyceps sinensis* polysaccharides and polypeptide extracts was obtained from Alpha healthcare, NZ. Epicor, a yeast glucan extract was obtained from Embria, USA. Glucagel, barley beta-glucan was obtained from DKSH, Germany. Non-extracted kelp (*Polygonum cuspidatum*) powder was from R&T Australia PTY and a seaweed extract rich in sulfated polysaccharide (fucoidan) was from Glenorie International, NZ. Vera Pure (Aloe vera) polysaccharides were obtained from Naturex, USA. Turmeric

extract (curcumin), Korean Ginseng root extract (*Panax ginseng*) and extract of Gotu Kola (*Centella asiatica*) were supplied by Phytomed, NZ. Lentinex was from GlycaNova, Norway.

#### **Human whole blood stimulation assays**

Heparinized human blood obtained from healthy volunteers was diluted in complete media (RPMI/10% bovine fetal calf serum/antibiotics; Invitrogen, USA) to a final dilution of 1/10. Diluted blood was cultured (37°C 5% CO<sub>2</sub> humidified incubator) with indicated concentrations of colostrum or immune stimulatory carbohydrates obtained from various commercial suppliers. All ingredients were prepared aseptically as a solution or suspension in complete medium. Cultures were maintained at 37 °C in a humidified, 5% CO<sub>2</sub> incubator for 72 hours. Cell-free supernatants were assayed for levels of stem cell related factors VEGF, GCSF, and IL-6 using flow cytometric bead array technology according to manufacturer's instructions (Becton Dickinson).

#### **Bone marrow viability, proliferation and differentiation assays**

Mouse femurs were purged with complete medium supplemented with 10<sup>-5</sup>M 2-mercaptoethanol to isolate bone marrow cells. Cells were cultured for 5 days at 1.5 x 10<sup>6</sup>/mL (37 °C humidified, 5% CO<sub>2</sub>) in medium or a dilution series of bovine colostrum. Multi-parametric flow cytometry was used to determine total cell viability, proliferative and differentiation status as well as CD34<sup>+</sup> cell survival. Cultured cells were washed into staining buffer (PBS/0.1% serum/0.09% NaN<sub>3</sub>) and then stained with amine reactive live/dead dye (aqua live dead; 1/1000) and DNA reactive dye Hoechst (10 µg/mL) (both from Invitrogen, USA) in the presence Lineage-APC, c-Kit-PE-Cy5 and CD34-FITC fluorescent antibodies (Becton Dickinson, USA). At least 100,000 events were acquired. Post acquisition analysis was performed using FlowJo (TreeStar, USA).

#### **Trial Formula**

Amounts of each ingredient to include in a trial formula to achieve bioactivity were chosen based on published feeding studies in disease models where possible [20, 21, 30-36]. For the mouse feeding study the ingredients were prepared as a suspension in water, according to the proportions in Table 2. The maximum amount of trial formula administered (TF Max) equated to a total 616 mg of ingredients containing 320 mg of colostrum. This was doubly diluted three times to the lowest dose of 40 mg. The maximum amount of ingredients in the top dose was limited by the ability to form a homogenous suspension that was fluid enough for oral gavage.

#### **Animal feeding study**

Mice (5 per group) were administered 1 mL of four decreasing amounts of the trial formula (TF Max, TF 1/2, TF1/4, TF 1/8) daily for 14 days between 12-4 PM. An equivalent amount of Alpha Lipid colostrum in the top dose of trial formula or water was administered for comparison. An oral gavage needle was used to administer the formulation. At all other times mice fed *ad libitum* on standard mouse chow. On day 14 mice were euthanized and a bone marrow cell suspension was prepared using both femurs from each animal for CFU assay and cell cycle analysis. Each mouse was analyzed

individually.

#### **Colony forming unit (CFU) assay**

Bone marrow cells ( $4 \times 10^4$  cells/1.1 mL) were suspended in methylcellulose medium containing optimal growth factors to enable the expansion and differentiation of progenitor cells into a range of lineages (MethoCult® GF M3434 methylcellulose medium with recombinant cytokines for mouse cells; Stem Cell Technologies, USA) and plated in 35 mm petri-dishes intended for progenitor cell enumeration (Stem Cell Technologies). The cells were cultured for 8 days in a humidified incubator at 37°C, 5% CO<sub>2</sub>. The colonies were visualized at 10X magnification using a microscope and the numbers of colonies were counted and expressed as number of CFU per  $4 \times 10^4$  cells.

#### **Bone marrow DNA content analysis**

At least  $5 \times 10^6$  bone marrow cells from each animal were fixed in 70% methanol and left overnight at 4°C. The following day, cells were washed into PBS by centrifugation and finally suspended in a solution of propidium iodide (Invitrogen, USA) at 50 µg/mL in PBS containing 1 µg/mL RNase A (Sigma, USA). Propidium iodide pulse width and area signals were collected using a Becton Dickinson LSR I flow cytometer from a minimum of 10,000 cells. Single cells were gated, and the proportion of cells that had greater than G1 phase (1N) DNA content based on propidium iodide fluorescence were determined (S/G2 cells).

#### **Pilot human study to determine stem cell mobilization**

Healthy volunteers (n=9) ceased consumption of other supplements and conventional medicines for 5 days prior to study. On the day of the study, subjects consumed the intended human dose (Table 2) of active ingredient (n=6) or placebo capsules (n=3) early in the morning (8 am) in a blinded fashion prior to the consumption of breakfast, to ensure fast adsorption. Venous blood was drawn from into heparin anti-coagulant tubes at pre-treatment, 1, 3, 6 and 24 hrs following consumption of placebo or active ingredient. Tubes were stored at room temperature until analysis. Cell staining (0.1 mL of blood/test) with panels of fluorescent monoclonal antibodies to detect stem cell subsets was performed. Cells were stained in TruCount™ tubes (Becton Dickinson), which contain a known number of fluorescent counting beads, using a lyse-no wash method to facilitate accurate enumeration. Red blood cells were lysed following staining with FACSlyse (Becton Dickinson). Approximately 500,000 events were acquired from each tube. Fluorescent antibody staining panels (all from Biolegend, USA) used were as follows: For hematopoietic stem cells: CD45-PECy7, CD184 (CXCR4) PE, HLADR-APC Cy7, CD34-APC. For mesenchymal/stromal cells: Lineage-FITC, CD309-PE, CD105-PerCP Cy5.5, CD34-PECy7, CD90-APC along with Hoechst nuclear dye. Samples were analyzed on a LSR II flow cytometer (Becton Dickinson). Stem cells subsets were resolved using multi-gate analysis (FlowJo) and the absolute counts of each subset were determined.

#### **Statistical analysis**

All data were analysed using Prism GraphPad (La Jolla, CA). Two group comparisons were assessed by Student's t test (parametric). p values < 0.05 were considered significant.

## RESULTS

GCSF (granulocyte colony stimulating factor) and IL-6 (interleukin-6) are important cytokines involved in stem cell release and maintenance of "stem-ness" or pluripotency as well as cell migration, whilst VEGF (vascular endothelial growth factor) is an important migratory and engraftment factor for stem cells at sites of tissue damage [37-40]. Peripheral blood from healthy human volunteers was used to screen for cytokine production, as it is a rich source of innate myeloid cells, which are also present in human intestinal lamina propria, a major cellular target for colostrum following consumption. Alpha Lipid colostrum was shown to induce GCSF, IL-6 and VEGF in a dose-responsive manner (Fig. 1), indicating that bovine colostrum can reliably induce cytokines that are known to be associated with increased bone marrow stem cell activities such as release from the stem cell niche, migration and engraftment.

To demonstrate that colostrum could influence stem cell-associated parameters at the cellular level, the viability, proliferation and differentiation of mouse bone marrow cultures following culture with colostrum was determined using multiparametric flow cytometry (Fig. 2). Viable cells were determined based on lack of amine-reactive dye uptake (Fig. 2a). In the absence of exogenous growth factors, colostrum improved the overall viability of the culture compared to medium alone at all concentrations tested (Fig. 2a). Associated with increased overall viability, the proportion of CD34<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup> stem cells in the bone marrow cultures also increased with increasing colostrum concentration (Fig 2b).

To determine the extent of cell division, cell cycle analysis was performed based on DNA content analysis. As cells divide the DNA content increases which corresponds to an increase in Hoechst DNA dye staining intensity. Cells with increased DNA content are referred to as S/G2 cells. The inclusion of colostrum was also associated with an increased proportion of dividing cells, as assessed by S/G2 DNA content analysis. Differentiation of mouse bone marrow cells was determined by upregulation of lineage marker expression on viable cells (Fig. 2d) and colostrum readily supported the expansion of more differentiated, lineage marker expressing cells with the highest concentration of colostrum tested showing the greatest effect (25 mg/mL). Taken together these results demonstrate that Alpha Lipid colostrum increases the viability, proliferation (expansion) and differentiation of precursor cells residing within the bone marrow and can also directly support CD34<sup>+</sup> stem cells, increasing the proportion of viable stem cells detected within day 5 mouse bone marrow cultures.

To establish whether immune stimulatory carbohydrates could synergise with colostrum for IL-6 and GCSF production, fucoidan, a seaweed extract rich in sulfated fucose was used as an example. Using human whole blood cytokine stimulation assays the individual bioactivities of fucoidan and colostrum were determined when used singly or in combination. Fucoidan was pre-determined to have a maximal *in vitro* stimulatory concentration of 100 µg/mL (data not shown) and was added to a suboptimal

concentration of colostrum (100 µg/mL; Figure 1). As shown in Fig. 3a, fucoidan and colostrum had synergistic activity for the production of IL-6 and GCSF. These findings support the hypothesis that immune stimulatory carbohydrates may be combined with colostrum to enhance the production of two key cytokines that relate to hematopoietic and mesenchymal stem cell activities.

Since there are many different sources of immune enhancing plant and fungal polysaccharides, and different types of extract that would also influence potency, a range of polysaccharides were sourced from commercial suppliers. Extracts were assayed across a dose-response range using the human whole blood IL-6 and GCSF release assay. The results are summarised in Table 1 and are expressed as fold changes relative to baseline readings and also as fold changes relative to levels induced with colostrum (1 mg/mL). The concentration range of ingredients associated with maximal *in vitro* activity for cytokine induction is also indicated, as a measure of potency. Based on these findings it was clear that certain plant and yeast carbohydrates were very potent inducers of IL-6 and GCSF release, being active at 1/10 of the concentration of colostrum required to achieve an equivalent response. To facilitate oral delivery of effective amounts, the more potent sources were selected for a trial combinatorial formulation.

Ingredients selected to supplement Alpha Lipid colostrum activity based on bioassay screening data were combined in a trial formulation detailed in Table 2. Amounts of ingredient to use were based on published feeding studies in animal disease models where efficacy was demonstrated against therapeutic endpoints. To confirm bioactivity, the ability of the trial formula to induce IL-6 and GCSF hematopoietic cytokines in human whole blood cytokine release assays was compared to colostrum alone. The results shown in Fig 3b demonstrates that the trial formula was more potent than an equivalent amount of colostrum for the induction of the stem cell related cytokines, achieving maximum stimulation at 1/10th of the amount of colostrum required to achieve a similar magnitude of response.

To test whether this formula could activate stem cell activity following oral consumption, an animal feeding study was conducted. Mice were fed the trial formula described in Table 2. As a comparison for TF max, an equivalent amount of colostrum was evaluated. Mice fed water served as an experimental control. At the end of 14 days of a daily feeding cycle the bone marrow cells were harvested for determination of the frequency of hematopoietic stem cells by CFU assay. This assay is a direct method for the enumeration of hematopoietic progenitor (stem) cells as it is based on the number of colonies that expand and differentiate from a single pluripotent stem cell. The results are shown in Fig. 4a, where the number of progenitor cells is expressed per  $4 \times 10^4$  bone marrow cells. It is clear that there were significantly more progenitor cells in animals fed the trial formulation at the highest dose (TF Max) than mice fed water or colostrum alone. Mice fed lower concentrations of the trial formula showed evidence of a reduced dose response, further suggesting that oral supplementation with the trial formula enhanced the number of stem cells in the bone marrow. Colostrum supplementation alone also lead to a smaller, but statistically significant increase in the number of progenitor cells compared to water alone fed mice.

As stem cells enter into the circulation to migrate to sites of repair, a homeostatic feedback system ensures there is continued replenishment within the bone marrow stem cell niche, which involves *in*



*situ* stem cell replication [41]. Analysis of bone marrow cells for mitotic cells, based on increased DNA content (S/G2 region) as shown in Fig. 4b demonstrated that a greater proportion of bone marrow cells were replicating in mice fed the highest dose of the trial formula compared to the other doses tested. A pilot human study was also undertaken to determine whether short-term changes in stem cell subset numbers in the peripheral blood could be detected following dietary supplementation with the trial formula. This activity, termed mobilization, is a pre-requisite in order for stem cells to reach the target organ or tissue. To assist the identification of normal fluctuations in stem cell numbers, placebo-fed subjects were also evaluated. Subjects ceased all other supplements for 5 days prior (washout period) and received the trial formula or placebo in a blinded fashion first thing in the morning and prior to breakfast to enhance gut exposure to the ingredients. Mesenchymal/stromal and hematopoietic stem cell numbers were determined in blood samples taken at 0, 1, 3, 6 and 24 hr following consumption of trial formula or placebo using a multi-parametric flow cytometry absolute counting method. The % change in cell numbers compared to baseline (T=0) levels determined for CD309<sup>+</sup> (VEGF receptor) stromal/mesenchymal stem cells and hematopoietic CD34<sup>+</sup> stem cells for all subjects is shown in Fig 4c. In the subjects fed the trial formula the majority of subjects (5/6) showed a greater range of change over the time points examined, as well as a greater number of changes (peaks and troughs) than seen in the placebo controls. Also, individual treated subjects showed a bi-phasic response e.g. (increase – decrease – increase) or (decrease-increase-decrease). None of these observed patterns were evident in the placebo controls. In spite of the small cohort size, the data overall clearly show that there were substantial changes (e.g. 100% and 60% increases) in some trial formula fed subjects compared to placebo controls. The largest changes were detected mostly around 3-6 hours. Some subjects showed marked decreases, taken as emigration of stem cells from the circulation to tissues or back to stem cell niches such as the bone marrow, whilst others show a marked increase. An increase in circulating stem cells can be interpreted as more cells being released from the bone marrow. Most of the changes in the 1-6 hour period were transient since they were not sustained at 24 hours. The transient nature of the response is consistent with an indirect activation of host immune tissue in response to a single bolus of an immunostimulatory composition.

## DISCUSSION

Normally stem cells are released under the influence of immunological stimuli, which establishes feedback loops that in turn recruit stem cell associated regenerative pathways. Hematopoietic and mesenchymal/stromal stem cells are key to these pathways since they are potent modulators of immune responses, as well as promoting tissue vascularization and preventing fibrosis [42-45]. Aging and disease influence the efficiency of these regenerative processes and there is increasing interest in developing ways to enhance these endogenous cellular regenerative pathways by nutraceutical means. Already it is well established that plant phytochemicals can effectively modulate oxidative stress pathways that contribute to cellular dysfunction, thereby supporting the body's repair mechanisms [46]. Furthermore, by using *in vitro* screening assays, medicinal plants extracts can be ranked for potency [47], increasing the likelihood that a therapeutic dose may be achieved following consumption.

Many therapeutic claims associated with plant-based nutraceuticals are developed around activities associated with parentally administered compounds, commonly using intraperitoneal and intravenous delivery routes. How these activities extrapolate to oral bioavailability is unclear. Here we were able to clearly demonstrate using a wide range of biological assays that bovine colostrum was able to stimulate the production of stem cell-related cytokines (IL-6 and GCSF) as well as growth factor, VEGF. Accordingly, colostrum induced stem cell activity *in vitro* and *in vivo* following oral supplementation. Given the pivotal role that IL-6 and GCSF play in enabling stem cell related activities [43], we exploited synergistic activity that we identified between natural extracts of immune stimulatory carbohydrates and colostrum to develop a trial formula which was effective in stimulating stem cells in an animal feeding study, supporting the rationale for this ingredient formulation. The inclusion of colostrum with immune stimulatory carbohydrates is important since it was observed that colostrum but not carbohydrates induced VEGF, a very important factor for mesenchymal/stromal cell activity. In agreement, a pilot human study also showed evidence that this trial formula modulated peripheral blood and hematopoietic and VEGFR<sup>+</sup> stromal stem cell numbers.

There is a large body of scientific data that supports the hypothesis that dietary supplementation with bovine colostrum can provide broad ranging immune modulation and have an impact on the severity of a range of disorders such as inflammatory bowel disease, liver disease, ulcerative colitis and infective diarrhea [30, 48, 49]. In contrast, the ability of bovine colostrum to specifically support stem cell associated regenerative pathways following consumption is not well established, although there is evidence that is indicative of these types of activity. For example, a major colostrum protein lactoferrin, as well as its proteolytic breakdown products, have been shown to enhance myelopoiesis when taken orally. This process depends on hematopoietic progenitor cell activities [50]. Further, oral dosing with bovine colostrum in a mouse model of cerebral ischemia was shown to have profound effects at limiting brain tissue damage, thus exhibiting neuroprotective activity, which was associated with reduced levels of serum inflammatory mediators [51]. Other evidence that colostrum may activate the stem cell niche and promote regeneration is suggested by studies on sports related injury and stress where endurance athletes are shown to have enhanced muscular-skeletal recovery when supplementing with bovine colostrum during training [52-54]. There are only a few other natural compounds and nutraceutical formulations that have been tested for stem cell-enhancing activity *in vivo* [55, 57]. Whilst stem cell mobilization has been described in these studies, the authors detect a wide variation in the extent of activity detected in individuals. However, such observations underscore the potential for natural compounds to promote regenerative activity.

## CONCLUSION

Considered together, the results from the mouse and human studies demonstrate that the presented Alpha Lipid colostrum/natural extract combination can stimulate an increase in the frequency of bone marrow progenitor cells, support self-renewal and promote release into the peripheral circulation. Furthermore, since this multi-ingredient combination exploits functional synergy between bovine colostrum and a range of immune stimulatory carbohydrates, it may achieve broader immunological

activity and at a dosage that is translatable into therapeutic benefit. Finally, these findings strengthen the hypothesis that inclusion of certain classes of functional foods in the diet can influence the cells that support healing and regenerative cellular pathways.

## **ABBREVIATIONS**

CFU colony forming unit

GALT gut associated lymphoid tissue

GCSF granulocyte colony stimulating factor

IL-6 interleukin 6

PBS phosphate buffered saline

VEGF vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

## **CONFLICT OF INTEREST**

New Image Group Ltd, a company specializing in developing and commercializing novel bovine colostrum-based nutraceuticals, sponsored this study. P. Lehrke is a fulltime employee of New Image Group. G. Webster was remunerated as a contract research scientist for the new product development program.

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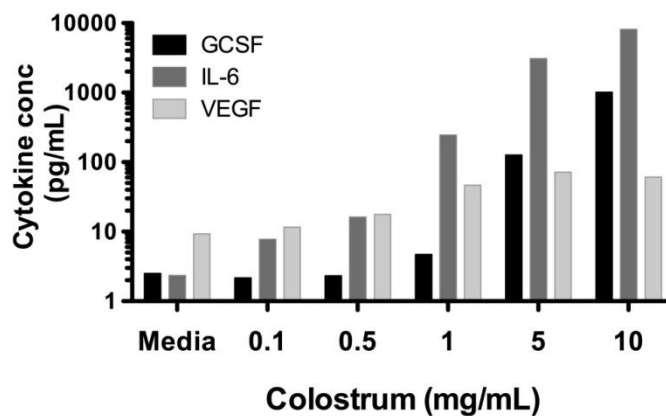
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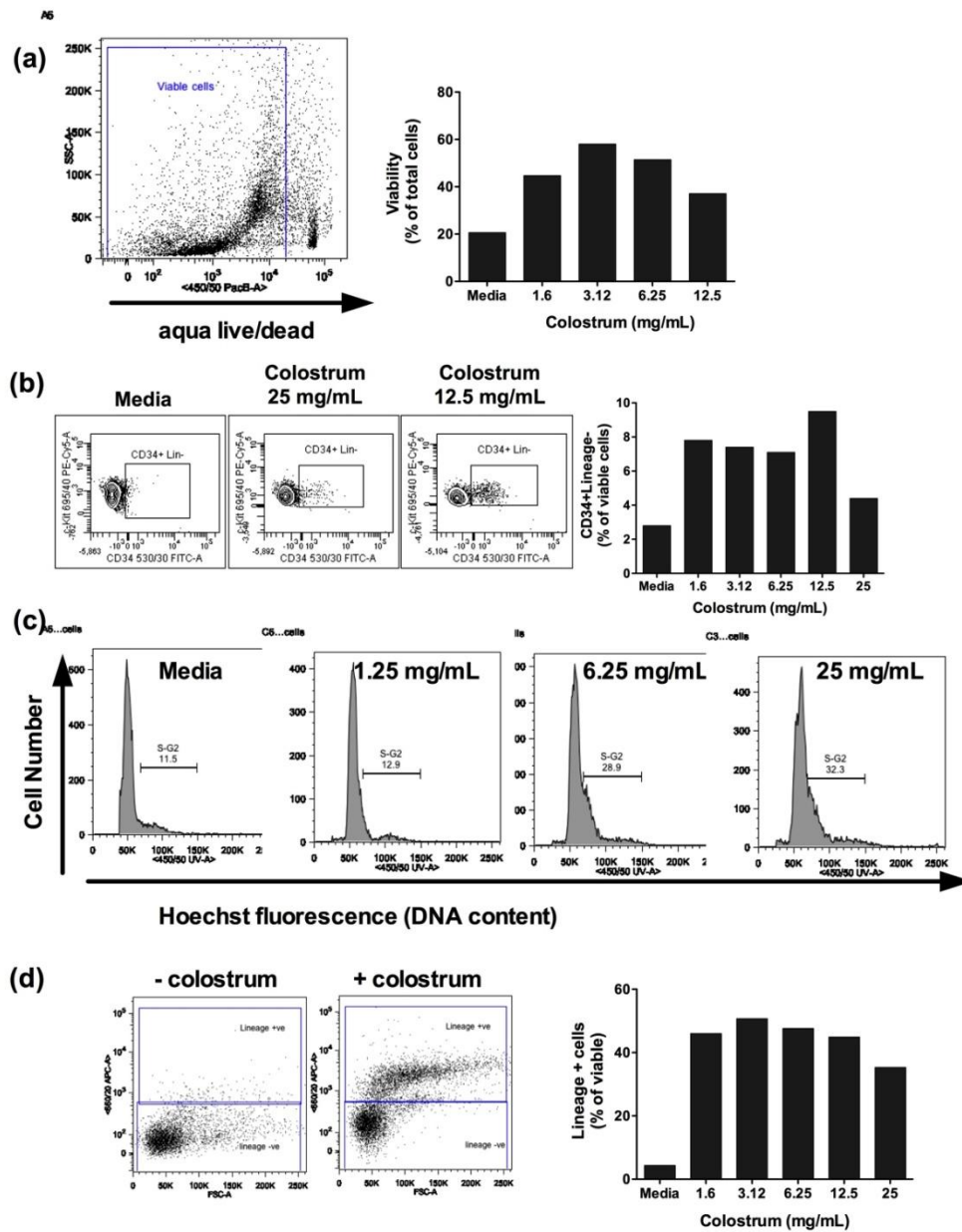
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## FIGURES



**FIGURE 1.** Production of GCSF, IL-6 and VEGF following 72 hr *in vitro* stimulation of human diluted whole blood cells with a titration of Alpha Lipid colostrum. Cytokines were quantified using analyte-specific flow cytometry bead array analysis. These results are representative of at least 3 independent experiments.

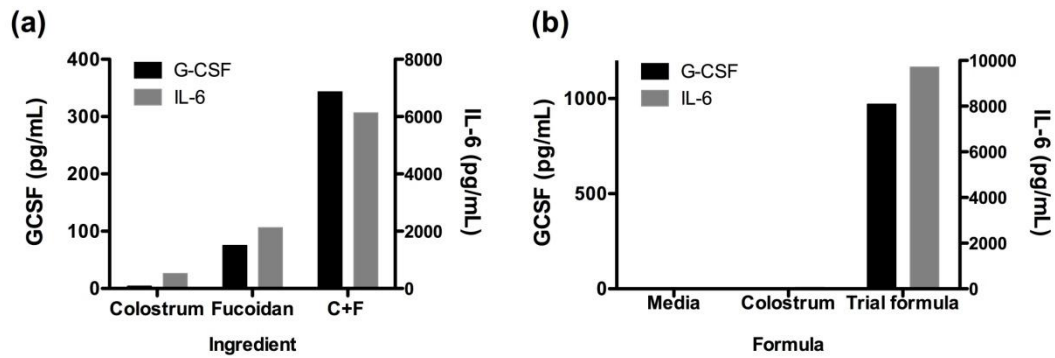
**FIGURE 2**



**Figure 2.** *In vitro* effect of Alpha Lipid colostrum on (a) mouse bone marrow viability (b) proportions of CD34<sup>+</sup> Lin<sup>-</sup> ckit<sup>+</sup> stem cells within the viable cell population (c) cell proliferation, as determined by DNA content analysis and (d) progenitor cell differentiation as determined by increased lineage marker expression after 5 days in cell culture.

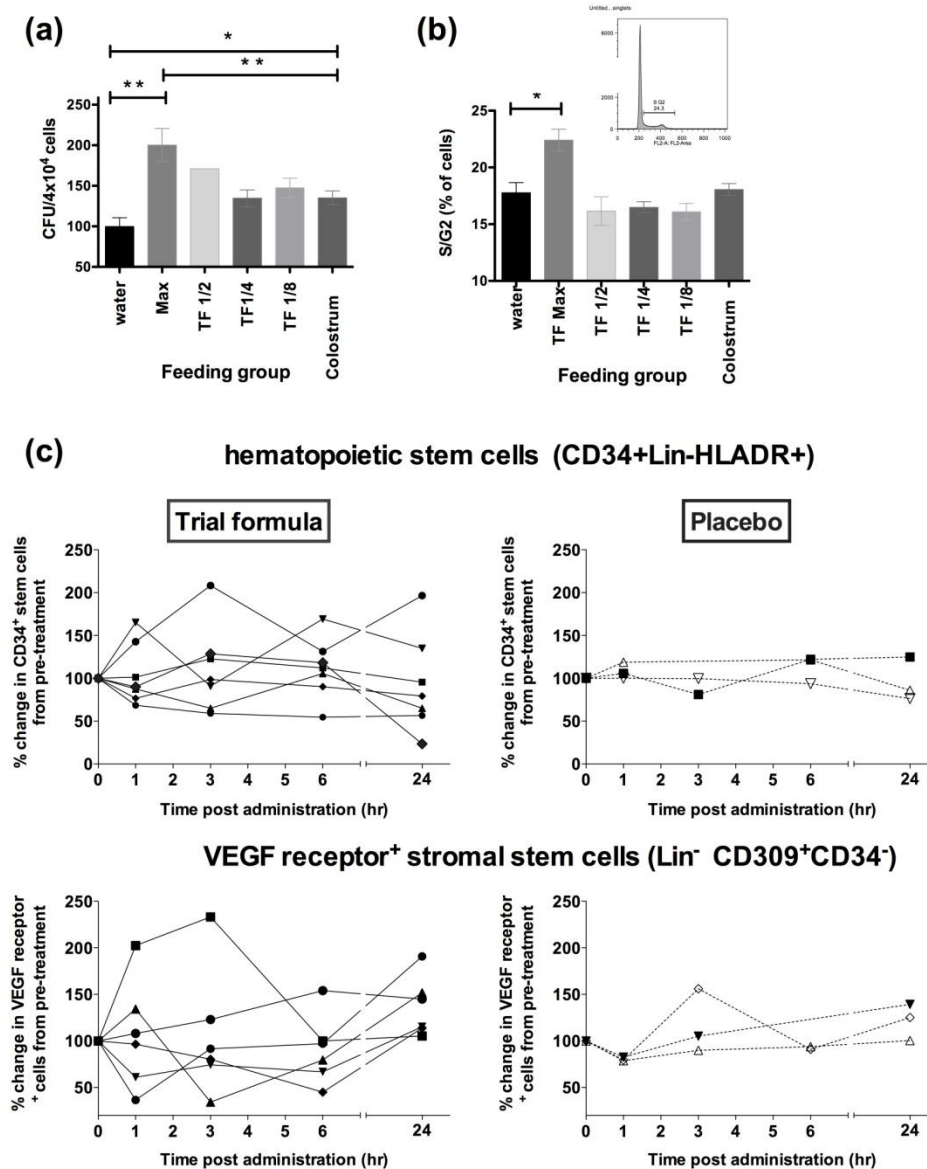


Figure 3



**Figure 3.** (a) Alpha Lipid colostrum bioactivity in a human whole blood G-CSF and IL-6 release assay is synergistically enhanced in the presence of fucoidan, an extract of sulfated fucose from kelp. The amount of G-CSF and IL-6 induced by 0.1mg/mL of colostrum or fucoidan alone and 0.1 mg/mL of each combined together is shown. (b) Alpha Lipid colostrum/carbohydrate trial formula (Table 2) is more potent than an equivalent amount ( $w/v$ ) of colostrum. Whole human blood was stimulated with 40  $\mu\text{g/mL}$  of colostrum or trial formula and G-CSF and IL-6 production was determined.

**FIGURE 4**



**Figure 4.** (a) Daily feeding of mice (N=5 per group) for 14 days with trial formula (described in Materials and Methods) induces a greater increase in the number of bone marrow progenitor cells (CFU) compared to mice fed an equivalent amount of colostrum alone, as determined in day 8 bone marrow CFU assays. (b) To determine the overall % of cycling bone marrow cells in trial formula, colostrum or water fed mice, bone marrow cells were processed and stained for DNA levels using propidium iodide for flow cytometric analysis. Dividing cells have an increased DNA content (S-G2) (b; insert). Mice fed the highest concentration of trial formula showed a significantly higher % of dividing bone marrow cells. Graphs represent mean  $\pm$  SEM (c). Consumption of trial formula by healthy subjects (n=6) induces changes in the numbers of circulating peripheral blood hematopoietic and mesenchymal stem cells compared to placebo fed subjects (n=3). Due to the differences in absolute counts of cells between individuals the changes in count are expressed as a % of the number obtained from T=0 samples. Statistical significance is indicated (Student t test; (\* P< 0.05; \*\* P< 0.01).

## TABLES

			Fold induction relative to unstimulated cells <sup>a</sup>		Fold induction relative to 1 mg/mL Alpha Lipid colostrum <sup>b</sup>	
Carbohydrate type	Ingredient	Conc for max stim mg/mL	GCSF	IL6	GCSF	IL6
β1-3:1-6 glucan	Shitake mushroom	1	100	1642	5.73	1.72
β1-3:1-6 glucan	Reishi mushroom	1	139	1774	7.94	1.86
β1-3:1-6 glucan	GanoPoloyC mushroom	0.1	75	1139	4.28	1.19
β1-3:1-6 glucan	Glugagel	0.1	1.67	170	0.09	0.17
β1-3:1-6 glucan	Lentinex	0.1-1	25	265	0.29	0.03
β1-3:1-6 glucan	Yeast extract	0.1	293	9626	3.48	56.99
Undefined	Scuterllaria	1	732	6130	41.75	6.40
Undefined	Fructus Jujube	1	107	2474	6.14	2.59
n-acetylated β-1,4-linked glucomannan	Vera	0.5-10	73	9626	0.87	1.33
sulfated fucose	Seaweed extract	0.1	75	846	0.15	0.50
sulfated fucose	Kelp	0.1	5	395	0.01	0.09
phytochemicals	Gotu Kola	10	ND	159	0.09	0.17
phytochemicals	Turmeric extract	5-10	6	376	0.26	0.41
phytochemicals	Ginseng	1/100	ND	4	0.03	0.23

**Table 1.** Bioactivity of various sources of immune enhancing polysaccharides in human whole blood stimulation assays for induction of GCSF and IL-6. The response is shown as fold stimulation relative to unstimulated cells<sup>a</sup> as well as to cells stimulated with Alpha Lipid colostrum at 1 mg/mL<sup>b</sup> to identify ingredients that are more potent on a weight basis than colostrum for cytokine induction.

<b>Trial formula (2 capsules)</b>
410mg Alpha Lipid colostrum
100mg Scuterllaria
100mg Fructus jujube berry
50mg Turmeric extract
50mg Yeast extract
20mg Seaweed extract
20mg L-Carnosine antioxidant

**Table 2.** Composition of trial formula of Alpha Lipid colostrum combined with immune stimulatory carbohydrate formula.

